Vol. XLI, No. 2 (April, 1960), was issued on 22.4.60.

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

VOL. XLI JUNE, 1960 NO. 3

BONE CHANGES ASSOCIATED WITH CORTISONE ADMINISTRATION IN THE RAT

Effect of Variations in Dietary Calcium and Phosphorus

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Received for publication December 8, 1959

The rat (Follis, 1951 ; Storey, 1960), unlike the rabbit (Sissons and Hadfield, 1955 ; Storey, 1957) and man (Reifenstein, 1958), does not develop osteoporosis following excess cortisone administration ; instead, dense metaphyseal bone forms. Experimental studies show that while cortisone inhibits growth in both the rat and rabbit the rate of bone resorption is significantly different. In the rat, resorption is delayed with consequent formation of dense bone whereas in the rabbit resorption is increased with development of osteoporosis. This difference in bone change may be associated with the remarkably adaptive calcium metabolism of the rat. Young rats can absorb and retain up to 98 per cent Ca from a low Ca diet (Fairbanks and Mitchell, 1936 ; Henry and Kon, 1939) ; they thus fail to develop typical rickets in the absence of vitamin D unless concomitant imbalance of dietary Ca and P be present (McCollum, Simmonds, Shipley and Park, 1921). Cortisone neither delays absorption of ^{45}Ca from the gut (Clark, Geoffroy and Bowers, 1959) nor inhibits the hypercalcaemia and associated changes of hypervitaminosis D (Wilson, Care and Anderson, 1957 ; Cruickshank and Kodicek, 1958). In contrast with the rat, studies in man show that during growth children only retain approximately one-quarter of the dietary Ca (Kinsman, Sheldon, Jenson, Bernds, Outhouse and Mitchell, 1939) and avitaminosis D will lead to the development of rickets.

In man the hypercalcaemia of hypervitaminosis D and sarcoidosis is restored to normal levels whereas that of hyperparathyroidism is unaffected by cortisone (Anderson, Dent, Harper and Philpot, 1954 ; Connor, Hopkins, Thomas, Carey and Howard, 1956 ; Verner, Engel and McPherson, 1958). Their results led Anderson *et al.* (1954) to suggest that cortisone may antagonize the action of vitamin D in promoting Ca absorption from the gut. Thus, as well as inducing osteoporosis of bone, cortisone also effects calcium metabolism in man whereas little significant effect has been observed in the rat.

In order to circumvent this exceptional ability of the rat to absorb calcium, in the present experiments during cortisone administration the dietary Ca and P has been lowered or the ratio altered. In these conditions the rat develops an osteoporosis similar to that seen in rabbits treated with cortisone.

MATERIALS AND METHODS

Two hundred and fifty two rats of both sexes were separated into groups ; each group was subdivided, one sub-group injected with cortisone acetate (40 mg./kg./day) and the other the control. Animals were weighed and killed at intervals until the 16th day when the remaining rats were killed and their bones split in the sagittal plane and examined macroscopically, then fixed in 10 per cent formol saline, decalcified and embedded in paraffin wax. Sections were cut at 5–7 μ and stained with Ehrlich's haematoxylin and eosin.

The basic diet consisted of variations of that described by Steenbock and Black (1925) : 78 per cent maize–20 per cent wheat gluten, 1 per cent NaCl and 1 per cent cod liver oil. The Ca and P level was of the order of 0·05 per cent calcium and 0·25 per cent phosphorus. Calcium as $CaCO_3$ and phosphorus in the form of NaH_2PO_4 were added in various amounts to the basic diet at the expense of maize so that different amounts and ratios of Ca and P were present in the individual diets. Animals were allowed water *ad libitum*.

RESULTS

Experiment 1 (180 *rats*)

The effect of different levels of balanced Ca: P in the diet was observed in 5 groups of 36 rats weighing 40–100 g. ; the following levels were used :—(i) 1·5 per cent Ca : 1·0 per cent P ; (ii) 1·0 per cent Ca : 0·6 per cent P ; (iii) 0·6 per cent Ca : 0·4 per cent P ; (iv) 0·4 per cent Ca : 0·25 per cent P ; (v) 0·25 per cent Ca : 0·25 per cent P.

Weight change.—Control animals gained most weight on 1·0 per cent Ca : 0·6 per cent P and least on the low calcium level—0·25 per cent Ca : 0·25 per cent P. With cortisone administration no group gained while groups on high or low Ca and P levels (i), (iv) and (v) lost appreciable weight (Fig. 1).

Macroscopic examination.—In the control groups bone trabeculae, shorter than those in animals on standard laboratory diet, were observed with high levels of balanced Ca : P (groups i, ii) ; although slightly shorter with intermediate levels of balanced Ca : P (groups iii and iv) they were considerably reduced with the low Ca : P diet (group v). With cortisone administration, only in the highest Ca : P group (i) did dense sclerotic bone form in any significant amount in the metaphysis of the femur and tibia. On intermediate or low balanced levels of Ca : P, metaphyseal trabeculae were reduced in amount.

Microscopic examination.—In the control groups at the end of 16 days, on the high Ca : P diets—groups (i) and (ii)—the epiphyseal cartilage is slightly narrower and metaphyseal trabeculae shorter and narrower than in animals on standard laboratory diet (Fig. 2). In groups (iii) and (iv) metaphyseal trabeculae are slightly shorter and thinner than in groups (i) and (ii), but bone growth continues relatively normally (Fig. 3). On the low Ca : P diet trabeculae are still shorter and resorption is increased.

In the cortisone treated group on the high Ca : P level there is a steadily increased density of metaphyseal bone until the 12th day ; by the 16th day this sclerosis is less obvious owing to the development of a few large marrow spaces (Fig. 4). In other groups bone becomes progressively less dense. On intermediate levels of balanced Ca : P long dense metaphyseal trabeculae remain in

the metaphysis—resorption is increased at bone margins compared with the previous group (Fig. 5). In the low Ca : P groups only a few metaphyseal bone trabeculae remain and resorption is occurring over a wider area than in the other groups.

Experiment 2 (48 *rats*)

Some unbalanced Ca : P ratios were added to the basic diet. Two extremes, high Ca : low P (1·5 per cent : 0·25 per cent) and low Ca : high P (0·05 per cent : 1·0 per cent) are described.

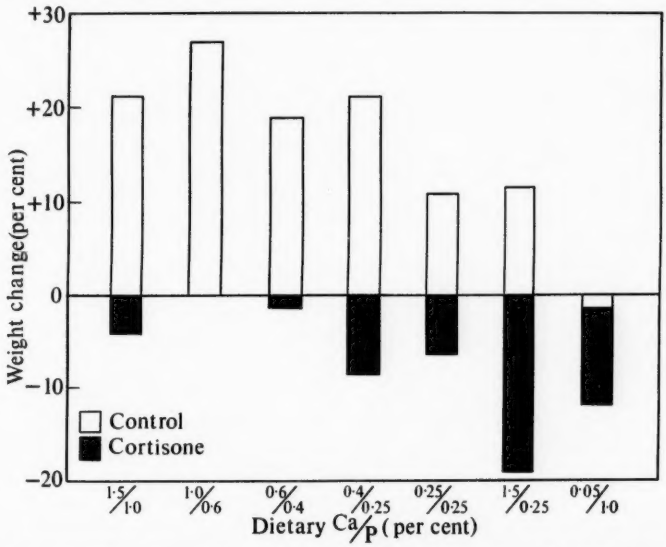


FIG. 1.—Diagrammatic representation of the weight change, expressed as the percentage change from the original weight, of groups of control and cortisone treated rats after 16 days on different levels and ratios of Ca and P. Control animals gained most weight on a balanced ratio of Ca : P with a calcium level from 1·5 per cent to 0·4 per cent. On low (0·25 : 0·25 per cent) or unbalanced ratios of Ca : P rats gained less; the group on low Ca : high P (0·05 per cent : 1·0 per cent) failed to gain weight. With cortisone administration animals on a balanced ratio of Ca : P with a calcium level from 1·5 per cent to 0·6 per cent lost least and those on unbalanced ratios of Ca : P lost most weight.

High Ca : Low P (24 *rats*)

Weight change.—Control animals gained considerably less weight (12 per cent) than rats on balanced and adequate Ca and P. When cortisone was administered rats in this group lost 19 per cent in weight, the greatest amount in any cortisone treated group (Fig. 1).

Macroscopic examination.—In the control group at the end of 16 days the epiphyseal cartilage is wider and metaphyseal trabeculae shorter than animals with balanced Ca : P in the diet. With cortisone administration the epiphyseal cartilage is narrow and little metaphyseal bone remains.

Microscopic examination.—As early as 4 days in the control group a widened osteoid border is visible on the endosteal surface near the metaphysis and by

eight days this is pronounced. At this time, while the metaphyseal region is narrow, the epiphyseal cartilage is irregularly widened in the zone of provisional calcification. After 16 days the cartilage is still irregularly widened and long thin metaphyseal trabeculae are lined by osteoblasts; wider layers of osteoid have now formed in the shaft (Fig. 6).

After 4 days with cortisone the cartilage is narrowed but less so than in other groups at this stage. By 16 days the cartilage is still narrower and only a few irregular metaphyseal trabeculae remain (Fig. 7). Although in some places trabeculae lined by osteoblasts show extremely thin margins of osteoid tissue, considerable resorption of the shaft is in progress.

Low Ca : High P (24 *rats*)

Weight change.—In contrast with other experiments, control animals failed to gain weight and with cortisone lost 12 per cent of their weight (Fig. 1).

Macroscopic examination.—In the control group after 16 days the width of the epiphyseal cartilage was similar to that in experiment 1 while the metaphysis was extremely narrow. In the cortisone treated animals the epiphyseal cartilage was narrowed, the metaphysis absent and the diaphysis extremely fragile.

Microscopic examination.—In the control group, after 8 days, the epiphyseal cartilage is slightly narrowed and metaphyseal trabeculae are reduced in length; a layer of osteoid extends down the endosteal surface of the shaft. At the end of 16 days metaphyseal trabeculae are reduced to an even narrower layer in which osteoblasts continue to lay down matrix (Fig. 8). The diaphyseal cortex, although lined by osteoid, is now thin.

EXPLANATION OF PLATES

FIG. 2.—Photomicrograph of section of the tibia showing the epiphyseal cartilage and metaphyseal bone after 16 days on a high balanced Ca : P diet (1·5 per cent Ca : 1·0 per cent P). Ehrlich's haematoxylin and eosin. ×100.

FIG. 3.—Photomicrograph of section of the tibia showing that the metaphyseal trabeculae are slightly shorter after 16 days on a balanced Ca : P diet (0·6 per cent Ca : 0·4 per cent P). H. and E. ×100.

FIG. 4.—Photomicrograph of section of the tibia after 16 days' cortisone treatment on a high balanced Ca : P diet (1·5 per cent Ca : 1·0 per cent P). The epiphyseal cartilage is narrow and wide bony trabeculae encasing calcified cartilage cores remain in the metaphysis. H. and E. ×100.

FIG. 5.—Photomicrograph of section of the tibia after 16 days' cortisone treatment on a balanced Ca : P diet (0·6 per cent Ca : 0·4 per cent P). Epiphyseal cartilage is narrow and only a few dense trabeculae remain in the metaphysis. H. and E. ×100.

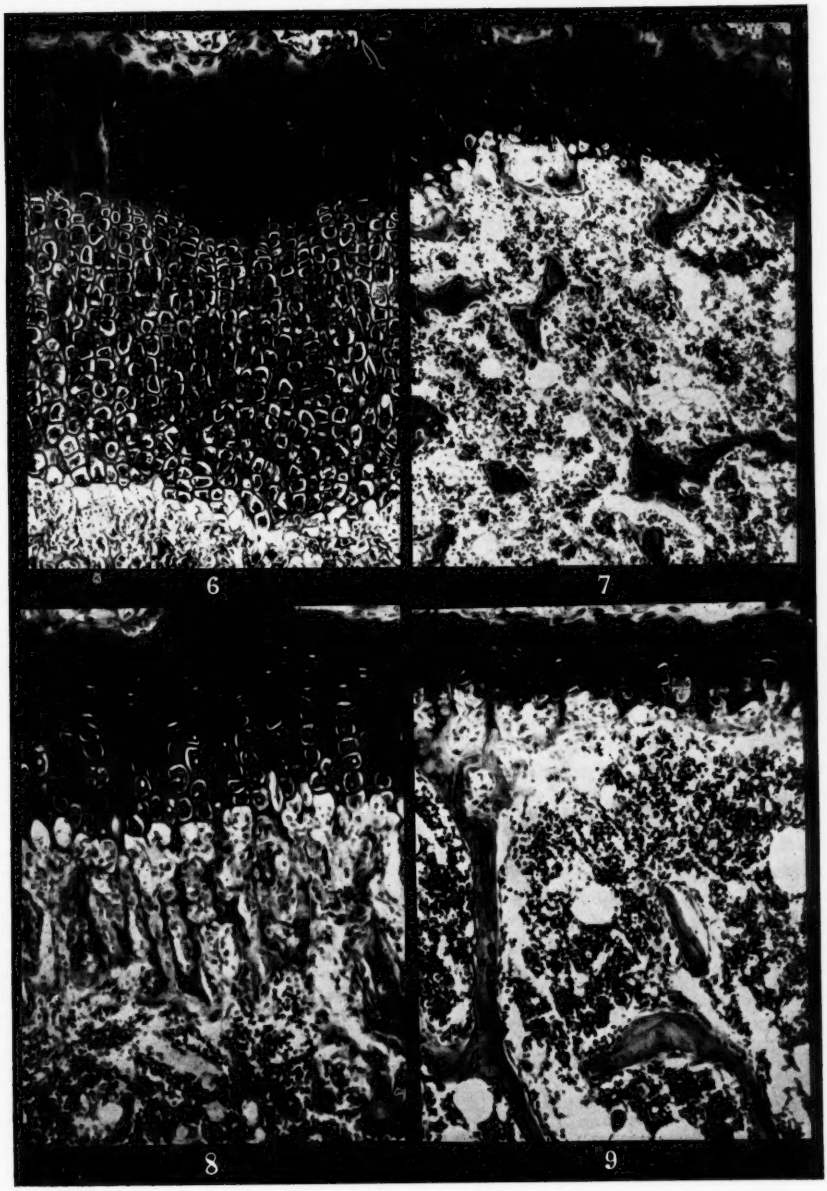
FIG. 6.—Photomicrograph of section of the tibia after 16 days on a high Ca : low P diet (1·5 per cent Ca : 0·25 per cent P). The epiphyseal cartilage is increased in width. H. and E. ×100.

FIG. 7.—Photomicrograph of section of the tibia after 16 days' cortisone treatment on a high Ca : low P diet (1·5 per cent Ca : 0·25 per cent P). The epiphyseal cartilage is decreased in width and only a few remnants of the metaphyseal trabeculae remain. H. and E. ×100.

FIG. 8.—Photomicrograph of section of the tibia after 16 days on a low Ca : high P diet (0·05 per cent Ca : 1·0 per cent P). The epiphyseal cartilage is of similar width to that in animals on a balanced Ca : P diet, but the metaphyseal trabeculae are shorter and resorption is increased. H. and E. ×100.

FIG. 9.—Photomicrograph of section of the tibia after 16 days' cortisone treatment on a low Ca : high P diet (0·05 per cent Ca : 1·0 per cent P). The epiphyseal cartilage is narrow and only a few trabeculae remain connecting the cartilage to the shaft. Resorption is greatly increased at the cartilage margin. H. and E. ×100.

Storey.



Storey.

In the cortisone treated group, after 8 days, the layer of metaphyseal bone consists of a few long interconnecting trabeculae. Along the shaft are areas where osteoblasts line trabecular margins ; in other areas resorption is progressing. After 16 days the epiphyseal cartilage is narrow, metaphyseal bone is absent and only a few trabeculae connect it to the diaphysis where numerous osteoclasts lie in lacunae (Fig. 9). Osteoid is not present at bone margins.

Experiment 3 (24 *rats*)

The effect of cortisone on a vitamin D deficient diet was tested in a group of 24 rats fed the basic diet without cod liver oil plus 1·5 per cent Ca : 1·0 per cent P. This ration was shown to be deficient in vitamin D by the production of rickets when NaH_2PO_4 was omitted from the diet.

Control bones were normal both macroscopically and microscopically while cortisone treated rats had dense metaphyseal bone after 16 days. This appeared considerably denser than the comparable group of rats with cod liver oil in the diet (experiment 1(i)).

DISCUSSION

With alteration of Ca and P levels in a basic Steenbock-Black diet the rate of growth varies, a maximum occurring with Ca : P of 1·7 : 1 with a Ca level of 1·0 per cent ; as the level of Ca and P is lowered or unbalanced, rats gain less until on a low Ca : high P diet they lose weight. Maximum rate of growth in these experiments occurred at a considerably higher Ca level than that usually regarded as being necessary for optimal growth, that is, 0·5 to 0·64 per cent Ca (Cox and Imboden, 1936 ; Toepfer and Sherman, 1936). This may be due to the stock animals being accustomed to a high level of dietary Ca and P and some time may be required for adaptation to low levels ; thus the optimal level of Ca for maximum rate of growth in these circumstances could well be higher than usual. Following cortisone administration rats fail to gain weight, those on adequate Ca and P losing least, those on inadequate or unbalanced Ca : P losing more.

Previous experiments have shown dense metaphyseal bone formation in cortisone treated rats (Storey, 1960) and the present study shows this to occur only with high balanced levels of dietary Ca and P ; with low or unbalanced levels bone becomes progressively rarefied. It should be noted that the level at which dense bone forms is higher than that usually required for optimal growth. This may be due, as pointed out above, to animals being accustomed to a high dietary level of Ca and P. The variation from dense to porotic bone is the result of changes in the relative proportions of the two processes responsible for remodelling of bone, formation and resorption. On high balanced Ca : P, endochondral ossification is delayed and a dense metaphysis forms, largely due to decreased extent of resorption of bone. As the dietary Ca : P level becomes lower, although some dense bone forms, bone is rapidly resorbed so that only remnants of wide trabeculae remain in the metaphysis. On the low Ca : high P diet a contributing factor in the development of osteoporosis with cortisone is the failure of growth, for on this diet control animals, in contrast to other groups, lost weight.

Albright (1942) supported by Sissons (1956), has proposed an anti-anabolic process to account for osteoporosis seen in Cushing's syndrome and that following cortisone administration (Sissons and Hadfield, 1955) ; that is to say, bone

growth is inhibited while resorption proceeds at the usual rate. While this may be true for man the present observations in rats show that the rate of removal of bone with cortisone is not invariable and depends on the level of dietary Ca and P. Thus factors modifying the rate of resorption, in addition to inhibition of bone matrix formation, are of significance in determining the degree and rate of bone change following cortisone administration.

Although, in bone, the histological picture of developing osteoporosis following cortisone treatment is similar in a number of animals it is probable that diverse mechanisms could lead to the same end result. Thus experimental osteoporosis in cortisone-treated rats fed abnormal Ca and P diets may not have a direct bearing on actual mechanisms involved in other species. However, in man, unlike the rat (Clark *et al.*, 1959), cortisone induces a significant deficiency of calcium which led Van Buchem (1959) to postulate that if this loss predominates over the anti-anabolic action on the osteoid matrix a picture of osteomalacia will arise. McLean and Budy (1959) have recently restated the proposition that Ca deficiency does not lead to " osteoporosis " but to " osteomalacia " where wide osteoid seams are characteristically visible. The present experiments show that cortisone does inhibit the formation of wide osteoid seams so that " osteomalacia " resulting from deficiency of Ca is not recognizable histologically since osteoporosis develops instead.

In addition to failure of osteoid tissue formation, resorption of bone is increased in cortisone treated rats fed unbalanced dietary Ca : P. The stimulus for this may possibly be related to activity of the parathyroid gland in maintenance of serum calcium levels. On Ca deficient diets, although resorption is increased, little new bone matrix forms during cortisone treatment. This combination of increased removal and decreased formation of bone leads to extreme rarefaction of the skeleton. Although it is not necessary to lower the dietary Ca to induce osteoporosis in rabbits with cortisone administration the process is, like that in the rat, greatly accelerated when diets are deficient in Ca. Indeed bone is removed at such a rate that multiple fractures occur within 12 days (Storey, 1960).

While compensatory parathyroid action may account for the increased resorption seen on calcium deficient diets, the bone changes with a high Ca : low P diet following cortisone treatment are more difficult to interpret. In young rats on this diet a temporary and modified form of rickets, similar to that described by Carttar, McLean and Urist (1950), develops despite the presence of vitamin D. Animals with cortisone on this diet are listless, eat little and lose more weight than other groups so that a number of factors contribute to the resultant osteoporosis. It is also worthy of note that the group on high balanced Ca : P without vitamin D, during cortisone treatment, developed sclerotic bone somewhat denser than that with vitamin D. Although this is surprising it may well be due, in the absence of vitamin D, to failure of the Ca mobilizing effect of vitamin D on bone (Carlsson and Hollunger, 1954). Further experiments to determine the interrelations between cortisone, parathyroid activity and vitamin D are indicated.

Investigation of many facets of the action of cortisone on bone, particularly with respect to experimental osteoporosis, is now possible in the rat. As a working hypothesis it is proposed that osteoporosis associated with cortisone administration results from a combination of two factors ; one being anti-anabolic in nature and inhibiting growth, the other " osteomalacic " and reducing calcium

absorption or retention so that compensatory bone resorption to maintain serum calcium levels occurs.

SUMMARY

The development of dense metaphyseal bone in cortisone treated rats occurs only on a high balanced dietary level of Ca and P: as the level is lowered, although some dense bone forms, it is resorbed rapidly and the metaphysis becomes rarefied; on unbalanced Ca : P ratios resorption predominates over formation so that bones rapidly become porotic. On these unbalanced ratios cortisone inhibits the development of wide uncalcified osteoid seams so that histological study of bone is no longer an indicator of abnormal calcium metabolism. Thus cortisone by inhibiting growth, in the presence of an increased stimulus for resorption due to abnormal levels of dietary Ca and P, induces osteoporosis in the rat similar to that seen in the rabbit.

This work was carried out under a grant from the National Health and Medical Research Council of Australia. The cortisone acetate was supplied by Boots Pure Drug Co. (Australia) Pty. Ltd.

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PURIFICATION OF RENIN BY ELECTROPHORESIS, ADSORPTION AND IMMUNOLOGICAL METHODS

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Received for publication October 20, 1959

MOST efforts to purify renin from crude renal extracts have been either by fractional precipitation usually with inorganic salts or organic solvents, or by adsorption methods, (Schales, 1942; von Euler and Sjöstrand, 1943; Katz and Goldblatt, 1943; Marshall and Wakerlin, 1948). The highest purity so far reported is 780 Goldblatt units/mg. dry substance by Haas, Lamfrom and Goldblatt (1953) who used an elaborate fractionation procedure of 10 steps. The fraction from step 5 was used as starting material by Passananti (1959) for ion-exchange chromatography; he achieved a 10-fold purification with a final activity of 43·2 units/mg. protein.

The present study describes initial purification by the method of Haas *et al.* (1953) up to step 6 and then further purification by electrophoresis in starch gel (Smithies, 1955), kaolin adsorption, or immunological precipitation of impurities by specific antisera. In some experiments an attempt was made to combine all these techniques. An account is also given of some of the immunological properties of the purified samples.

METHODS

Fractional precipitation.—The renin was prepared from 44 kg. of pig kidneys by the method of Haas *et al.* taken as far as step 6 (Table I). The activity of the product was 80 units/ml. (5·0 mg. protein/ml.). It was stored at − 20° in 0·015 M pyrophosphate buffer at pH 5·3.

Bio-assay of renin.—This was carried out in rabbits and standardized against a renin sample kindly provided by Dr. Haas. (Nairn, Fraser and Chadwick, 1959).

Protein nitrogen estimations.—The micro-Kjeldahl method was used routinely. It was replaced by the more sensitive method of Fels and Veatch (1959) whenever the protein concentration was very low as in the final purification steps.

Starch gel electrophoresis.—Smithies' method was slightly modified as follows: The trough for the gel measured 30 × 4 × 1·25 cm. and the slot for the renin sample 3·5 × 1·2 × 0·3 cm. Step 6 renin was concentrated × 10 to 800 units/ml. (50 mg. protein/ml.) by evaporation and 1 ml. of the concentrate was introduced into the slot. Electrophoresis was carried out at 250 V for 48 hr. at 2°, using pyrophosphate buffer (0·02 M, pH 6·5). Because of the unusually prolonged electrophoresis, two separate bridge buffer solutions were needed to maintain a constant pH in the starch gel. For elution after electrophoresis, the gel was immediately inverted into a second trough $\frac{3}{4}$ the depth of the first, and the protruding gel (i.e. the lowermost $\frac{1}{4}$ of the original gel) was cut away for staining with Naphthalene Black 10B (George T Gurr). The rest of the gel was immediately transferred to the deep freeze and stored at − 20° for 24 hr.; elution was unsatisfactory without this step. The following steps were carried out at 0°–2°. The gel was cut transversely into 0·5–2·0 cm. strips; the narrower strips were taken where highest renin activity was expected from comparison with previous runs. Such comparisons between gels were made by reference to the stained slices. The strips were eluted with buffered saline (pyrophosphate, pH 6·5, 3 ml./1 cm. strip). Two thirds of the saline was used for initial disintegration of the gel by stirring with a glass rod and the

mixture was left to stand for 24 hr. to complete the extraction of the renin. The eluate was separated from the gel by passage through a small Buchner funnel and the gel fragments were washed twice with the remaining third of buffered saline. Any starch particles reaching the eluate were removed by centrifugation.

Kaolin adsorption.—An elaboration of the method of McEwen, Harrison and Ivy (1939) was used. Light kaolin (B.D.H.), heated for 4 hr. at 700° in a furnace to remove any nitrogenous impurity, was mixed with step 6 renin at pH 3·5 to form a 10 per cent suspension. This and subsequent steps were carried out at 0°–2°. The suspension was agitated for 1 hr. and left to stand for not less than 4 hr. to complete the adsorption. The deposit after centrifugation was washed several times (usually 4) with buffered saline (pyrophosphate, 0·02 M pH 3·5) until no protein could be detected in the washings. Then the renin was eluted from the kaolin by treating with buffered saline (pyrophosphate, 0·05 M, pH 7·3) to make a 10 per cent suspension. This was again agitated for 1 hr., left to stand for not less than 4 hr. and centrifuged to obtain the purified renin in the supernatant.

Immunological procedures

Preparation of antisera.—Two antisera were prepared: one against homogenates of pig kidney cortex in rabbits ; the other against step 6 renin in hens.

The pig kidney cortex homogenates (10 per cent suspension in physiological saline) were prepared from fresh kidneys from which pelvis and as much medulla as possible had been removed with scissors. Three adult rabbits were each given 3 ml. of the suspension in Freund's adjuvant, excluding *Mycobacterium tuberculosis*, in 3 intramuscular depots ; the injections were repeated after an interval of 2 months. At monthly intervals after this, two boosting doses of 2 ml. of the suspension were given, without adjuvant, intraperitoneally and the rabbits bled 6 days after the last injection. The serum (AK) with the highest precipitin titre was used in subsequent experiments. This serum, diluted 1/5, gave a positive ring test with a 1/8,000 dilution of centrifuged supernatant of the kidney suspension ; there was no antirenin activity.

Antirenin sera were prepared in six 2 kg. hens : (*a*) Three hens were each given 90 units of step 6 renin in 6 ml. physiological saline intraperitoneally, daily for 6 days, and the course repeated after a month. A third course was given after another month interval, the three final injections being intravenous. Serum obtained from one of the hens, 3 days after the last injection, showed a satisfactory antirenin titre (28 units/ml.); this serum, diluted 1/5, precipitated with step 6 renin at 1/50 dilution. The antibody response in the other 2 hens was poor. (*b*) In 3 hens, an adjuvant method using acid alum precipitation of the antigen was used (Wright, Green and Kanode, 1954). Basal immunity was induced by intravenous injection of 175 units of step 6 renin precipitated with alum : the renin activity was not reduced by the precipitation. Three further courses of injection, without alum precipitation, were given at monthly intervals as in (*a*), but a useful antibody titre was obtained in one hen only : the antirenin activity was 5 units/ml. and the serum, diluted 1/5, precipitated with step 6 renin at 1/200. The antirenin sera prepared by methods (*a*) and (*b*) (AR/28 and AR/5 respectively) were used for immunological investigation of the purified samples of renin.

It is interesting that in all hens, further immunization resulted in a reduction in antirenin titre while the precipitin titre either remained the same or increased.

Immunological analysis of renin samples.—This was carried out largely by Mansi's (1958) modification of the Ouchterlony technique using 1·0 × 4 mm. wells, 2·5 mm. apart, in gels made on standard microscope slides. This micro-method was preferred because of great scarcity of materials for some of the tests. Simple diffusion in columns (Oudin, 1952) was also tried for comparison with earlier analysis with rabbit antiserum (Nairn *et al.*, 1959) but was abandoned because the hen antisera induced milkiness of the agar, which obscured the precipitation lines.

Removal of precipitating impurities in renin samples.—The combination of pig renin with antirenin prepared in the dog, rabbit or rat, is non-precipitating (Lamfrom, Haas and Goldblatt, 1954). Using similar methods we found that the antirenin in the hen antiserum (AR/5) was also non-precipitating with pig renin. This was demonstrated by separating precipitate from supernatant in step 6 renin/antiserum mixtures and recovering the major part (60–70 per cent) of the antirenin from the supernatant by the alkaline dissociation method. Even the purest preparations of renin we have studied include, as impurities,

pig kidney proteins against which precipitating antisera may be prepared. Such antisera (AK and AR/5) were used to remove precipitable impurities from step 6 renin and electrophoretically-purified renin. The precipitation was carried out at optimum proportions, determined by trial and testing the supernatants by gel diffusion. In practice, the renin sample and the anti-serum were incubated in a water bath at 37° for 30 min., cooled in ice for not less than 30 min., and centrifuged for 15 min. at 10,000 r.p.m. When the antirenin sera were used for precipitation there was some associated loss of renin activity from the sample. Removal of precipitinogens results in a purification of the renin sample with respect to pig kidney protein though only at the cost of contamination with the rabbit or hen serum. For some immunological studies of renin this contamination should be unimportant.

Recovery of renin from rabbit and hen sera supernatants.—After removal of precipitable impurities by the specific antisera, a proportion of the renin in the supernatants was recovered by the kaolin adsorption method already described. This also reduced the contamination by the rabbit or hen antisera. In some experiments, with the object of minimizing contamination of the final renin samples by rabbit or hen serum proteins, the antisera were extracted with kaolin before they were used for precipitation. This procedure was later abandoned as it had the effect of reducing the antibody titre of the hen sera. Instead, the recovered renin samples at pH 7·3 were again shaken with kaolin and centrifuged; this removed a further proportion of the contaminant hen serum protein without material effect on the renin concentration.

RESULTS

The results of the initial purification of renin by fractional precipitation are given in Table I.

TABLE I.—*Purification of renin (method of Haas, Lamfrom and Goldblatt* (1953))

Step	Procedure	Yield (Units of renin)	Purity (Units/mg. dry wt.)
1	Water, ammonium hydroxide and benzene extraction with autolysis	164,000 (166,000)	0·037 (0·044)
2	Ethanol fractionation	142,000 (137,000)	0·86 (0·92)
3	Sodium tungstate precipitation	— (133,000)	— —
4	Calcium chloride dissociation and ammonium sulphate fractionation	50,000 (91,000)	3·6 (3·6)
5	Acetone fractionation	49,000 (87,000)	8·4 (9·4)
6	Ammonium sulphate fractionation and hydrochloric acid precipitation	32,000 (66,000)	14 (25)

The figures in brackets are those expected from the results of Haas *et al.* using the present weight of pig kidneys (44 kg.) as starting material.

The purity of the step 6 renin was 14 units/mg. dry weight., or 16 units/mg. protein. Our yield was less than that of Haas *et al.* because of a poor recovery from steps 3–4. The sodium tungstate precipitation is a difficult step; even after repeated pilot experiments to determine the optimum amount of tungstate, recovery of renin has been unsatisfactory. This may be attributable to slight excess of tungstate or somewhat excessive autolysis at step 1 with consequent extraction of undesirable substances: in either case the recovery of renin at steps 3–4 may be reduced (Haas, personal communication).

Electrophoresis

Fifteen electrophoretic experiments were carried out; a typical pattern and histogram of the observed renin distribution is given in Fig. 1. The material

eluted from the 0·7 cm. strip of gel at the peak of renin activity contained 40 units of renin in 60·5 μg. protein, a purity of 660 units/mg. This 41 fold purification of the step 6 renin was the best obtained in the series. The average purity at the peaks of the fifteen runs was 370 units/mg. protein (range 260–660). For the most part, the concentration of renin around the peak had a roughly normal distribution with a small deviation away from the origin in the direction of flow. The small peak in the figure at 8·5 cm. from the origin was not always observed and is probably not significant.

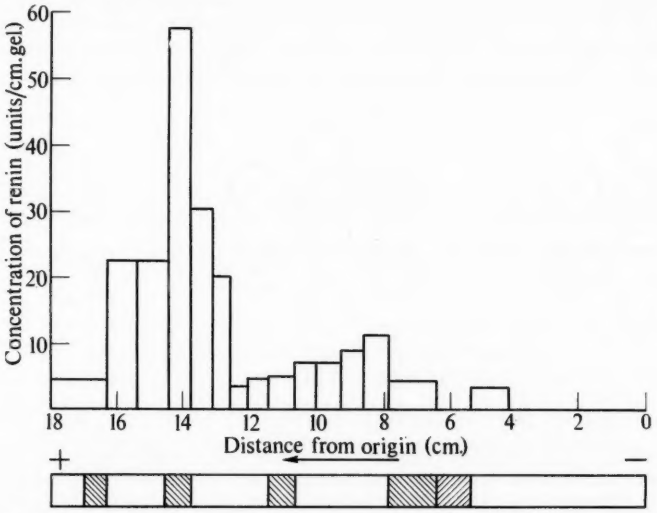

FIG. 1.—Histogram of renin concentration in starch gel after electrophoresis of step 6 renin. The diagram below is from the stained slice of gel to show the corresponding protein pattern.

On an average, the total recovery from the gels was only about 10 per cent of the renin applied. The reason for this is not clear: it might have been due to irreversible adsorption by the starch or to denaturation during electrophoresis.

In order to obtain sufficient material for subsequent manipulations, the purest renin samples were combined with eluates of lower purity from either side of the peak. Eluates with a purity less than 40 units/mg. protein were rejected; the final combined sample had a purity of 160 units/mg., *i.e.* 10 times that of the step 6 renin.

Kaolin adsorption

The recovery of renin from step 6 starting material varied from 60–90 per cent in different experiments, with a 10–14 fold purification. The renin obtained had a purity of 160–225 units/mg. protein.

Immunological studies

The number of specific precipitin lines formed by the various samples of renin against the AK or AR/5 sera are recorded in Table II, and illustrated in

Fig. 2. Non-specific lines also given by the pre-immune control sera, have been excluded. The gel diffusion analyses were sometimes difficult to interpret because of non-specific opalescent patterns produced by the electrophoretically-purified renin and by the hen serum. Slight contamination by starch may have been responsible for the non-specific reaction by the renin sample but we have no explanation for the hen serum reaction. Simple measures such as modification of pH failed to prevent the opacities and repeated analyses were required to obtain legible results.

The rabbit serum (AK) showed 3 sharp specific lines against the centrifuged supernatant of the original antigen (pig kidney cortex homogenate). One line of the 3 gave the reaction of identity with the single line formed between AK and step 6 renin. The antigenic component responsible for this line was not demon-

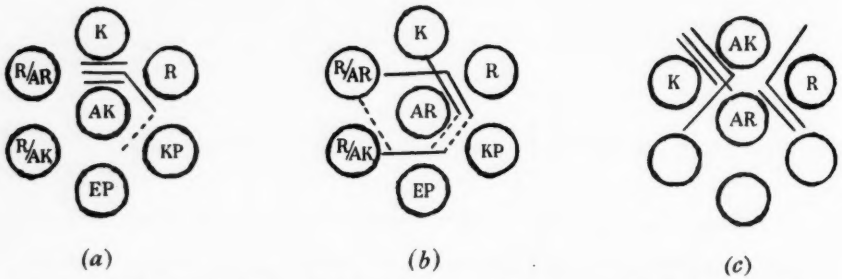

FIG. 2.—Gel diffusion analysis of renin preparations. Abbreviations are given in Table II.

(*a*) No lines between anti-kidney serum (AK) and pooled electrophoretically-purified renin (EP) or immunologically-purified renin (R/AK and R/AR). One feint line against kaolin-purified renin (KP) and the corresponding sharp line against step 6 renin (R) show reaction of identity with one of the three lines between AK and kidney cortex homogenate (K).

(*b*) The single line between antirenin serum (AR) and K shows reaction of identity with a line produced by R, KP, and EP, but not with the line against R/AK. There are two lines against R and neither are present against R/AR ; the common line is absent against R/AK.

(*c*) Line between K and AR shows identity with one of the lines against AK ; the line between R and AK shows identity with one of the lines against AR.

strated in the other renin preparations tested except for the kaolin purified sample which gave only a faint line.

The hen serum (AR/5) showed 2 sharp specific lines against the original antigen (step 6 renin). One of the lines gave the reaction of identity with the single line formed between AR/5 and cortex homogenate. The same 2 lines (both faint), were also given by kaolin purified renin. With the electrophoretically-purified material, one line only was present and this was scarcely perceptible in the peak samples even at a renin concentration of 40 units/ml. Similar results were obtained when AR/28 serum was substituted for AR/5 though the lines were less sharp.

Comparison of the two sera, AR and AK, (Fig. 2c) gave confirmatory results and some additional information : 1 of the 3 lines formed between the cortex homogenate and AK gave the reaction of identity with the single line against AR ; 1 of the 2 lines formed between step 6 renin and AR gave the reaction of

TABLE II.—*Purity and Immunological Gel-Diffusion Analysis of Different Samples of Renin*

	Renin sample*							
		Electrophoretically purified (EP)			Immunologically purified with			
	Fractionated Step 6 (R)	Peak samples	Pooled sample	Kaolin purified (KP)	AK serum Step 6 renin (R/AK)	AR 5 serum Step 6 renin (R/AR)	AR 5 serum Pooled EP renin	Pig kidney cortex homogenate (K)
Purity (units/mg. protein)	16	260–660 (mean 370)	160	225	165†	70†	25†	Negligible (0·1 u/mg.)
Specific gel diffusion bands at 16–24 hr.								
Rabbit serum, anti-pig kidney cortex (AK)	1	0	0	1 (faint)	0	0	0	3
Hen serum, anti-renin (AR)	2	1 (faint)	1	2 (faint)	(1)‡	0	0	1

* Samples diluted to contain 10 units renin/ml. for immunological analysis.

† Renin recovered by kaolin adsorption after removal of precipitinogens, but still contaminated with antiserum.

‡ Specific line probably present but largely obscured by a non-specific band and appearing as an incomplete splitting from this band.

identity with the single line against AK. This explains the ability of AK serum to neutralise one antigen as shown in Fig. 2b (AR against R/AK cf. R).

Immunological purification.—One of the precipitinogens in step 6 renin was removed, without any reduction in renin activity, by precipitation with AK serum at optimum proportions (1 ml. serum : 20 units renin). This precipitinogen was presumably a minor antigen in the cortex homogenate against which the AK serum was prepared ; the precipitating proportions are unrelated to the high titre observed against the major antigens.

AR/5 serum had an antirenin titre of 5 units/ml. and, when it was used for immunological purification, the pressor activity of the renin samples was reduced by this amount. However, the serum had the advantage that it could be used to remove both precipitinogens in step 6 renin. The precipitating proportion chosen was 1 ml. AR/5 serum : 40 units renin ; this proportion was the optimum for only one of the precipitinogens. No attempt was made to complete the removal of the other by precipitation because the extra serum required would have neutralised nearly all the renin ; but, as Table II shows, removal of this precipitinogen was apparently completed during the recovery of the renin by the kaolin adsorption method. For pooled electrophoretically-purified renin, the precipitating proportions were 1 ml. serum : 15 units renin.

The chemical purity of all 3 samples, after immunological precipitation, was low because of the contaminating antiserum. Recovery of the renin in the supernatants by the kaolin adsorption method reduced this contamination and further purification was obtained. In each case the actual yield of renin from these procedures was about half the theoretical yield. It is clear from Table II that the chemical purity of the final samples, because of residual antiserum contamination, is less than that of kaolin-purified step 6 renin, but the precipitinogens have been removed and the purity with respect to pig kidney protein may reasonably be assumed to be greater.

DISCUSSION

The fractionation method of Haas *et al.*, is a reliable method of obtaining a good yield of purified renin. The tungstate precipitation step is the only one presenting any real difficulty ; our yield at step 4 was relatively poor. Step 6 renin is excellent starting material for further purification procedures as the yield at this stage is still good and the renin stable ; it may be safely stored at pH 5·3 (pyrophosphate buffer, 0·015 M) at −20° for long periods.

Purification by kaolin adsorption provides a remarkably simple way of obtaining a high yield of renin (up to 90 per cent) with a purity 10 or more times that of the starting material. The method could presumably be applied profitably at any stage in the preparation of renin. It has been of great value in the present experiments in recovering renin from supernatants after immunological precipitation of impurities. The loss of antibody in the hen serum on preliminary absorption with kaolin was an unexpected finding which merits further study.

Starch-gel electrophoresis was an unsatisfactory procedure for the preparation of pure renin. Whilst very high purities were obtained with peak samples, the recovery was only about 10 per cent of the starting activity and even this necessitated extremely careful elution. The method cannot be recommended, therefore, and for future experiments along these lines we should prefer to try separation by means of columns of modified cellulose which have given promising results for other enzyme purifications (see Hill, Kimmel and Smith, 1959).

The immunological purification achieved its purpose of removing precipitinogens from step 6 renin and electrophoretically-purified renin, without serious loss of renin activity. Although the samples, even after kaolin recovery, were still contaminated with the antisera, the presence of such heterologous protein should not impair their usefulness for other immunological studies. For example, the non-precipitating samples from the pooled electrophoretically-purified renin would be valuable for " blocking ", in specificity tests, in the histological localization of renin with fluorescent antibody (Nairn *et al.* 1959). Such immunologically pure material has not so far been available.

The immunological studies may not be exhaustive because of the difficulty in reading diffusion analyses in small gels when non-specific opacites are present ; but they appear to be conclusive in the sense that the failure of the purified samples to produce lines has been confirmed repeatedly. Nevertheless, just as AR/5 serum contains antibodies which cannot be demonstrated in AK serum, it is possible that an antiserum against one of the purer renin preparations would reveal antigens not detected by AR serum. That a complex system of antigens does exist in pig renin purified by fractional precipitation has been revealed by the numerous precipitin lines given by rabbit antirenin serum (Nairn *et al.* 1959). Whether these antigens are all included in the two distinct reactions with the present AR hen serum or whether their identification has been obscured by non-specific reactions has not yet been decided.

In the process of purification of renin it is clear that some kidney antigens have been lost. Step 6 renin contained 2 demonstrable antigens other than renin ; one at least of the precipitinogens was removed by electrophoretic purification and the other by precipitation with a specific antiserum. The single line between the cortex homogenate and antirenin serum is interesting since it indicates selection of a particular precipitinogen in the crude kidney material, during the preparation of step 6 renin.

A useful practical aspect of the immunological study is that the number and intensity of precipitin lines were reliable criteria of purity of the renin samples. The gel diffusion method with such antisera would always provide a simple, rapid and highly economical means of testing renin samples at different stages of purification.

SUMMARY

Renin initially purified from pig kidneys by the fractional precipitation method of Haas *et al.* taken as far as step 6, was further purified by electrophoresis in starch gel, kaolin adsorption or immunological precipitation of impurities by specific antisera. These techniques were combined in some experiments.

Antisera, (AK) prepared in rabbits against pig kidney cortex homogenate or (AR) in hens against step 6 renin, contained precipitins against pig kidney protein impurities in the renin samples. The AR serum also showed antirenin activity; the renin/antirenin complex was non-precipitating. The antisera were used for immunological analysis, by gel diffusion, of the different renin preparations. The step 6 renin had a purity of 16 units/mg. protein and showed two precipitin lines when tested immunologically against AR serum. The purest renin sample, 660 units/mg. protein, was obtained by electrophoresis and showed only one faint precipitin line with AR serum. Immunologically purer preparations were obtained by removal of the precipitinogens by means of the antisera. Although purified with respect to the pig kidney protein, these preparations were less pure chemically owing to contamination by the rabbit or hen serum proteins. The contamination was reduced by final recovery of the renin by the kaolin adsorption method.

The gel diffusion technique with such antisera provides a simple, economical method of testing the purity of renin preparations.

We wish to thank Prof. J. S. Young, Prof. W. O. Kermack and Prof. A. Macdonald for helpful advice and criticism.

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THE GROWTH CYCLE OF ECHO VIRUSES IN TISSUE CULTURE.

II. RATE OF ADSORPTION OF ECHO VIRUS TYPES 1, 2, 7 AND 11 TO MONOLAYER CULTURES OF MONKEY KIDNEY CELLS.

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Received for publication October 20, 1959

In an attempt to explain the reasons for the differences in plaque producing potential with regard to speed of production and plaque size between ECHO virus types 1, 2, 7 and 11, Sommerville, McIntosh and Carson (1958) described the growth cycle of these viruses in terms of their maturation and release from infected monkey kidney monolayers. No correlation was found, however, between these characteristics and the differing plaque producing properties of the viruses. In view of the probability that some sequence of events within the growth cycle was responsible, attention was focused on the possibility that the explanation might lie in variations in the rates of attachment of the viruses to susceptible cells. This paper contains the results which were obtained from measurements of the rate of attachment of these viruses to monkey kidney cells and explains the reasons for the differences in plaque producing potential.

MATERIALS AND METHODS

Viruses

The virus stocks used were the same as previously described (Sommerville *et al.*, 1958). One member of each ECHO plaque group was chosen as follows: ECHO 1 (Farouk), ECHO 2 (Cornelis), ECHO 7 (Wallace), ECHO 11 (Gregory). Each was purified by passage 3 times at limiting dilution. ECHO 2 was in its 13th monkey kidney passage and the remainder had been passed 11 times.

Growth medium

This consisted of Hanks' balanced salt solution 93·5 per cent, 0·5 per cent lactalbumin hydrolysate, 5 per cent calf serum and 1 per cent sodium bicarbonate (4·4 per cent W/V).

Maintenance medium

Stationary tube cultures of monkey kidney cells were maintained in Earle's saline 89·5 per cent, 0·5 per cent lactalbumin hydrolysate, 5 per cent calf serum and 5 per cent sodium bicarbonate (4·4 per cent W/V).

Preparation of monkey kidney monolayers

Rhesus monkey kidneys were trypsinized by a modification of the Bodian (1956) method. The cells were dispersed in either 6 oz. "medical flat" bottles with screw tops or in 12 × 100 mm. test tubes with rubber bungs. Confluent monolayers were produced within 7–10 days, usually with a change of growth medium half way.

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EXPERIMENTAL

Three different methods were used to measure the rate of attachment of virus to cell.

Measurement by estimation of the rate of disappearance of virus from fluid overlay

Confluent monolayers in 6 oz. " medical flat " bottles were washed thoroughly and infected with a dose of each ECHO virus containing 1 ID_{50} (50 per cent infectious dose) per 1000 cells of the monolayer. This was suspended in 10 ml. of maintenance medium. The bottles were incubated at 37° and agitated in 2 planes every 10 min. Infectivity titrations were performed in test tube monolayers of cells from the same monkey kidney using 0·5 $\log_{10}$ steps and 5 tubes per dilution. The titres were read microscopically at 4 days. The titre of virus in the supernatant fluid was estimated half-hourly for 4 hr. by withdrawing 0·3 ml. samples for titration of infectivity.

Controls to obviate loss of virus by adherence to the glass surface were provided by inoculating identical clean sterile bottles which had no cells with an identical virus dose. These were treated in the same way as the " test " bottles ; 0·3 ml. samples were withdrawn half-hourly and titrated for infectivity in monkey kidney cells.

An average of the results which were obtained from 2 identical experiments with each virus is shown graphically in Fig. 1, and selected parameters by which the adsorption rate of each virus may be characterized are recorded in Table I. Adsorption of virus to the glass bottles may be discounted because in each experiment a remarkably constant value was obtained during the repeated sampling of the control bottles. Great care was taken to agitate the bottles at regular intervals and in two planes to keep unattached virus evenly dispersed.

TABLE I.—*Properties of ECHO Virus Types* 1, 2, 7 *and* 11 *and of Poliovirus type* 1 (*Mahoney*) *Derived from Adsorption Rates to Monkey Kidney Cells in Monolayers.*

Virus	Average adsorption time (min.)	Adsorption at 30 min. (per cent)	Adsorption at 60 min. (per cent)	Maximum adsorption (hr.)	Maximum adsorption (per cent)
ECHO 7	9	81	81	2·5	90
ECHO 11	12	80	84	2·0	85
ECHO 1	12	79	90	2·0	93
ECHO 2	90	19	35	3·0	72
Polio. 1	7·5	91	92	1·5	92·5

Although the figures which were calculated from the curves depicted in Fig. 1 for ECHO types 1, 7 and 11 differ slightly, the adsorption curves of these viruses are strikingly similar in shape. So far as the slope of the curve is concerned ECHO type 2 is unique. In the 3 similarly shaped curves there is an initial period of extremely rapid attachment of about 30 min. duration. The average adsorption time, representing the time at which 50 per cent adsorption has occurred varies from 9 to 12 min. Thereafter each curve flattens as the maximum adsorption point is approached. This is reached between 2·0 and 2·5 hr. and the maximum amount of virus adsorbed varies from 85 per cent for ECHO 11 to 93 per cent for

ECHO 1. These 3 viruses behave very similarly to poliovirus type 1 (Mahoney), the corresponding figures for which are appended to Table I.

ECHO type 2 has very different properties by this method of examination. The initial period of rapid adsorption is prolonged to 2·5 hr. before the curve shows any evidence of levelling (Fig. 1). The average adsorption time is 90 min. and maximum adsorption is achieved in 3 hr. when 72 per cent of the virus has become attached to the monolayer.

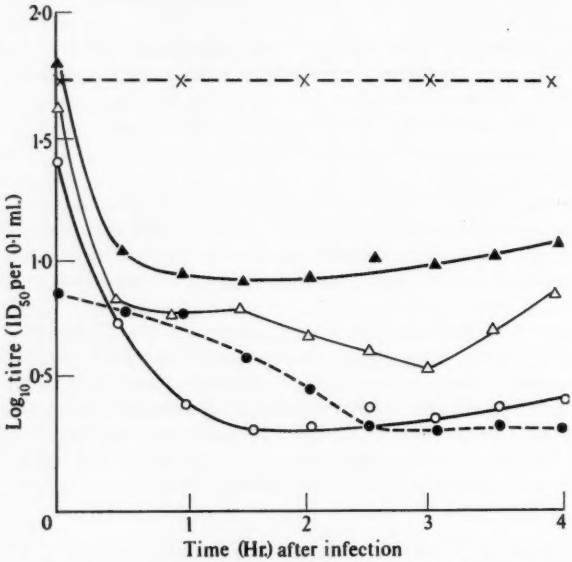

FIG. 1.—The rates of attachment of ECHO virus types 1, 2, 7 and 11 to monolayers of monkey kidney cells measured by the amount of free virus in the supernatant fluid at varying times after infection. The dose used was approximately 1 ID_{50} per 1000 cells for each virus.

×- - - -× Blank control
○——○ Echo 1
●- - - -● Echo 2
△——△ Echo 7
▲——▲ Echo 11

Measurement by counting the number of micro-plaques produced with increasing time of exposure of cells to virus

This technique is based on the micro-plaque method for enumerating ECHO virus particles described by Sommerville (1959). The experiments were performed in test tube monolayers of monkey kidney cells. The monolayers were washed 3 times with phosphate buffered saline (pH 7·2) and infected with approximately 50 plaque forming units of each virus in 0·1 ml. maintenance medium. The tubes were incubated at 37°. At 15 min. intervals groups of 5 tubes were washed vigorously 5 times to remove unattached virus and were then inoculated with 1·0 ml. of maintenance medium. This procedure was continued at 15 min.

intervals for 2 hr. The tubes were incubated for 36 hr. and examined microscopically. An average micro-plaque count was obtained from each group of 5 tubes and the results were plotted graphically. The technique was repeated twice for each virus in turn.

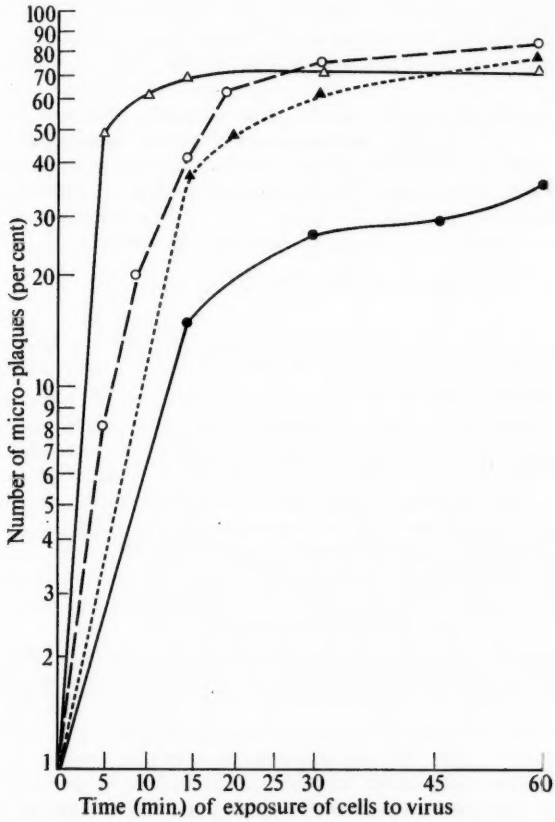

FIG. 2.—The rates of attachment of ECHO virus types 1, 2, 7 and 11 to monolayers of monkey kidney cells measured directly by the micro-plaque technique. The dose was approximately 50 PFU for each virus.

○- - - -○ Echo 1
●———● Echo 2
△———△ Echo 7
▲- - - -▲ Echo 11

Each virus is shown in Fig. 2 to adsorb directly to a monolayer of susceptible cells at a different rate. The rates can be arranged in a descending gradient as can the curves in Fig. 2 from left to right. This gradient is identical with that formed by the differences in the speed with which plaques are formed and the

TABLE II.—*Properties of ECHO virus types* 1, 2, 7 *and* 11 *derived from micro-plaque Estimations of the Adsorption Rates to Monkey Kidney Monolayers.*

Virus	Average adsorption time (min.)	Adsorption at 30 min. (per cent)	End of rapid adsorption period (min.)
ECHO 7	4·5	94	15
ECHO 1	15	88	30
ECHO 11	16	77	30
ECHO 2	20	69	60+

size of plaque eventually produced by the method described by Hsiung and Melnick (1957). These results are supported by the calculated parameters of adsorption expressed in Table II. The exception to this generalisation is ECHO 2 which will not produce plaques by Hsiung and Melnick's method, although this virus clearly takes its place at the lower end of the adsorption gradient and probably therefore appears in the plaque gradient as follows :—

ECHO 7, ECHO 1, ECHO 11, (ECHO 2).

Measurement by observing the length of time required following variable exposure to a high concentration of virus for the cells to show 50 *per cent degeneration.*

These experiments were performed in tube cultures of monkey kidney cells. The tubes were emptied of medium, washed thoroughly with phosphate buffered saline and inoculated with 0·1 ml. of maintenance medium containing 4 ID_{50} of virus per cell of the culture. The tubes were incubated at 37° and at 15 min. intervals 5 tubes were washed 5 times with phosphate buffered saline to remove unattached virus. 1·0 ml. of maintenance medium was added and incubation at 37° continued. The time required for the appearance of 50 per cent degeneration was observed by microscopic examination every 12 hr. and these times were plotted graphically against the duration of initial exposure to the virus.

This procedure was repeated in turn for each virus.

The results which were obtained by this method in which 4 ID_{50} of virus per cell of the culture was allowed to adsorb to monkey kidney monolayers in test tubes are shown in Fig. 3. The curve for each virus follows the same general pattern with a more or less steep initial sloping portion and a long horizontal " tail ". The initial slope covers samples during the first hr. of adsorption. Variation in the virus dose failed to produce alteration in the sloping portion of the curves. The greatest slopes were found with ECHO types 2 and 11. In each instance the fact that the graphs become horizontal after 1 hr. of adsorption indicates that a greater adsorption time fails to decrease the time required for the virus to destroy 50 per cent of the cells.

These indirect adsorption rates also give a direct correlation with the plaque producing properties of the viruses in that the plaque gradient is identical with the gradient of time taken to produce 50 per cent cell destruction.

DISCUSSION

Although a positive correlation with the plaque forming ability of the viruses under investigation can be obtained with either the microplaque method or the " indirect " method just described, some other factor must be responsible for the

complete inability of ECHO type 2 to produce plaques under agar by Hsiung and Melnick's (1957) method. The possible factors involved are now open to investigation by using the micro-plaque technique and form the subject of a separate communication (Sommerville, 1960).

In considering the " indirect " adsorption results just described, it is important to remember that this technique will measure only the cumulative effect of a number of factors which have already been measured individually for each virus by Sommerville *et al.* (1958). When these factors which are involved in the ECHO virus growth cycles and adsorption rates interact in the " indirect " measuring experiment, however, an exact replica of the process by which plaques are formed is produced. The results obtained in this way with ECHO virus type 2 indicate that not only does it adsorb more slowly and with less efficiency to susceptible cells than the others but it also produces plaques more slowly than the

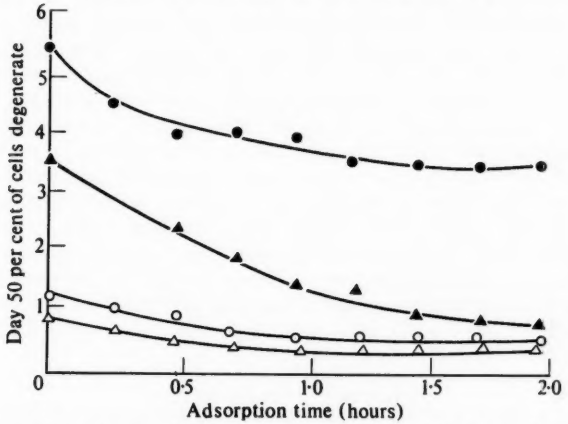

FIG. 3.—The rates of attachment of ECHO virus types 1, 2, 7 and 11 to monolayers of monkey kidney cells measured indirectly as the time required for 50 per cent of the cells to become degenerate after exposure to each virus. A dose of 4 ID_{50} per cell was allowed to adsorb for various times.

other viruses and is in some way prevented from doing so at all when the factors involved in plaque production by the method of Hsiung and Melnick (1957) are operating under test conditions.

SUMMARY

The rates at which ECHO virus types 1, 2, 7 and 11 adsorb to monolayer cultures of monkey kidney cells have been measured by 3 different methods. By using a micro-plaque method in which the number of virus particles adsorbing with increasing time of exposure was recorded, and also by using an " indirect " method in which the production of degeneration following variable exposure was

timed, the already well known difference in plaque producing potential of these four viruses can be explained in terms of adsorption rate differences.

I would like to express my gratitude for financial assistance to the Advisory Committee for Medical Research and to the Council of the National Fund for Poliomyelitis Research ; I am indebted to H. G. Carson, F.I.M.L.T. for excellent technical assistance.

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SOME FACTORS AFFECTING PLAQUE FORMATION BY ECHO VIRUS TYPE 2 IN MONKEY KIDNEY CELLS

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Received for publication October 20, 1959

THE demonstration by Hsiung and Melnick (1957) of differences in the plaque producing ability of ECHO viruses under agar in monkey kidney cells allowed the classification of these agents into four broad groups. One group, consisting of ECHO types 2, 3, 5 and 6 would not produce plaques under standard conditions in which the viruses were allowed to adsorb to monkey kidney cells for one hour before covering with a nutrient overlay containing neutral red and agar. All other ECHO viruses produced plaques under these conditions.

During the investigation of the reasons for the differences in plaque producing potential of a selected member of each plaque group ECHO type 2 was found by Sommerville (1959,1960) to produce microplaques in tube cultures using a fluid overlay medium. The supposition was made that production of " standard " plaques under agar must be suppressed therefore by one or more of the components of the overlay system. In this paper the components of the overlay have been examined in turn and evidence is produced to demonstrate that neutral red under certain circumstances is inhibitory to plaque production with ECHO virus type 2.

MATERIALS AND METHODS

Virus

The strain of ECHO type 2 (Cornelis) was in its 13th monkey kidney passage and had previously been passaged 3 times at limiting dilution.

Tissue cultures

The experiments were conducted in freshly trypsinized rhesus monkey kidney cells distributed in 12 × 100 mm. test tubes. Confluent monolayers were produced in 7 days.

Media

Growth medium (GM) consisted of Hanks' solution 93·5 per cent with 0·5 per cent lactalbumin hydrolysate, 5 per cent calf serum and 1 per cent sodium bicarbonate (4·4 per cent).

Maintenance medium (MM) consisted of Earle's saline 89·5 per cent with 0·5 per cent lactalbumin hydrolysate, 5 per cent calf serum and 5 per cent sodium bicarbonate (4·4 per cent).

Both media contained penicillin, streptomycin and mycostatin.

EXPERIMENTAL

A comparison of the media used by Hsiung and Melnick (1957) for the production of a " standard " plaque and by Sommerville (1959) to prepare micro-plaques

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reveals that in both the concentrations of Earle's saline, antibiotic mixture and calf serum are identical. The differences are summarised in Table I.

TABLE I.—*Differences between Components of "Standard" and Micro-plaque Media*

Constituents	Concentration in medium	
	"Standard plaque" (per cent)	Micro-plaque (per cent)
Sodium bicarbonate . .	0·225 g.	0·22 g.
Neutral red	1·6 mg.	—
Phenol red	—	2·0 mg.
Agar	1·35 g.	—

Phenol red is incorporated in the micro-plaque medium as a guide to pH only. Micro-plaques are enumerated microscopically and lack of staining is not required for their differentiation.

The micro-plaque technique was used in the experiments to be described. In each a small number of micro-plaque forming units (MPFU) of ECHO type 2 virus was adsorbed to monkey kidney cell monolayers for a standard time of 1 hr. Unadsorbed virus was removed by washing and the cells were covered with MM containing one of the components from the "standard" plaque overlay in varying concentration. In this way each of the differences between the "standard" and micro-plaque methods was examined in turn for its effect on suppression of micro-plaque production by ECHO virus type 2.

The effect of various sodium bicarbonate concentrations

Approximately 50 MPFU of ECHO 2 in 0·1 ml. of diluent was inoculated into each of 30 test tubes containing monolayers of monkey kidney cells. Unadsorbed virus was removed after 1 hr. at 37° by washing 3 times with phosphate buffered saline (PBS) at pH 7·2. The tubes were then treated in groups of 5 and to each group was added 1·0 ml. per tube of standard MM altered only in respect of the concentration of sodium bicarbonate. This varied from 0·022 g. per cent to 0·704 g. per cent and increased two-fold for each group of 5 tubes. Ten tubes which had been similarly inoculated were used for virus controls and these were given 1·0 ml. of standard MM containing the normal sodium bicarbonate concentration of 0·22 g. per cent.

The tubes were re-stoppered, incubated at 37° at 5° from horizontal and examined microscopically at 36 hr. when the number of micro-plaques was counted.

The results obtained are shown in Table II. At the normal sodium bicarbonate concentration of 0·22 g. per cent the estimated 50 MPFU per tube produced an average of 48 micro-plaques in the control tubes. The number of micro-plaques countable at the two lowest concentrations of bicarbonate is very small, probably due to the extremely low pH at 36 hr. The medium was a bright yellow colour, many cells were degenerate and many had detached from the glass. Similar, but slight, degenerative changes were noticed when the bicarbonate concentration was increased to 0·088 g. per cent. At the highest concentration the medium was light purple in colour and all the cells were degenerate due to the excessively high pH. Within the normal range of bicarbonate concentration the micro-plaque count was uniform and not significantly different from the virus control count.

TABLE II.—*The Effect of Varying Concentrations of Sodium Bicarbonate on Micro-plaque Production by ECHO type 2 Virus*

Concentration of $NaHCO_3$ in normal maintenance medium (g. per cent)	Average* micro-plaque count
0·220 (virus control)	48
0·022	3
0·044	3
0·088	28
0·176	55
0·352	56
0·704	Cells degenerate

* Average figure obtained from 5 identical experiments.

The effect of various concentrations of neutral red

In these experiments 40 MPFU of ECHO 2 were added to each of 40 tubes. The tubes were arranged in groups of 5 with 10 controls. After washing each group had 1·0 ml. of MM added containing a concentration of neutral red (Neutral Red—Fluorchrome, Gurr) varying from 0·25 mg. per cent to 3·0 mg. per cent. To each of the 10 control tubes 1·0 ml. of normal MM was added. The tubes were incubated for 36 hr. at 37° at 5° from horizontal and the micro-plaque count was then recorded.

TABLE III.—*The Effect of Varying Concentrations of Neutral Red on Micro-plaque Production by ECHO type 2 Virus*

Concentration of neutral red in normal maintenance medium (mg. per cent)	Average* micro-plaque count
0 (virus control)	30
0·25	16
0·5	19
1·0	7
1·5	0
2·0	4
3·0	0

* Average figure obtained from 5 identical experiments.

The results are recorded in Table III. The average number of micro-plaques produced in the virus control was lower than anticipated at 30 but at this level was sufficiently high to show differences with varying concentrations of neutral red. A progressive fall in the average count is evident until a concentration of 1·5 mg. per cent was reached when complete suppression of micro-plaque formation occurred. At a concentration of 3·0 mg. per cent the cells became rather toxic without showing definite necrosis. The cell sheet was disrupted to the extent that each cell had separated from its neighbours although individual cells retained their normal shape. The absence of micro-plaques at this concentration may reflect depression of cellular activity rather than a specific effect of the dye on virus reproduction. At all other concentrations the appearance of the cell sheet was completely normal apart from variable intensity of staining by the dye.

The effect of the addition of agar

The third possible difference between the " standard " and micro-plaque techniques was that agar is added to the overlay for the production of " standard "

plaques. In this experiment 40 MPFU of ECHO type 2 in 0·1 ml. MM were added to each of 40 tube monolayers of monkey kidney cells. After 1 hr. at 37° and removal of unattached virus by washing, the cells in 20 tubes were covered with 1·0 ml. MM containing 1·35 per cent agar (Difco-Bacto). This was allowed to solidify and the tubes were incubated at 37° for 36 hr. when the micro-plaques were counted. The 20 control tubes received an inoculum of 1·0 ml. of normal MM from which agar was omitted.

TABLE IV.—*The Effect of agar on the Formation of Micro-plaques by ECHO type 2 Virus*

Concentration of agar in normal maintenance medium (g. per cent)	Average* micro-plaque count
0 (virus control)	33
1·35	30

* Average figures obtained from 5 identical experiments.

The results are shown in Table IV. The addition of agar made no difference to the micro-plaque count nor did it affect the ease with which micro-plaques could be enumerated.

DISCUSSION

From a consideration of the results expressed above it is clear that neither the differences in concentration of sodium bicarbonate between "standard" and micro-plaque technique nor the presence of 1·35 per cent agar in the former accounts for the inability of ECHO type 2 virus to produce plaques by the "standard" technique.

It is clear, however, that neutral red has a profound effect upon plaque production by this virus in that a decrease in plaque numbers occurs with increasing concentrations of the dye until complete suppression of micro-plaque production is achieved when the concentration of neutral red is equivalent to that used in the production of the "standard" plaque. The degree of suppression is approximately proportional to the amount of neutral red present.

A similar experience is recorded by Waterson (1958) who found that neutral red had a very variable effect upon the plaque count produced by fowl plague virus in chick embryo cell cultures. If the neutral red was incorporated into the agar overlay the cells were either destroyed before plaques could be produced or the plaque count was unexpectedly depressed. In this instance the plaque count reduction was proportional to the quantity of neutral red and Waterson suggested that the cause was probably an impurity rather than the neutral red itself.

These observations add further support to the suggestion that in the production of "standard" plaques neutral red should be omitted from the agar overlay until plaques have developed. They can then be differentiated from the surrounding normal tissue by diffusion of a small amount of subsequently added neutral red through the agar layer.

SUMMARY

By means of a micro-plaque technique, the effect of various alterations in the composition of the overlay medium on the production of plaques by ECHO virus

type 2 has been measured. Alteration in the concentration of sodium bicarbonate within the physiological range or the addition of 1·35 per cent agar had no effect on the production of plaques whereas a progressive diminution in plaque count leading to complete suppression of plaque production was produced by the addition of increasing quantities of neutral red to the overlay medium.

I am grateful to the Advisory Committee for Medical Research and to the National Fund for Poliomyelitis Research for grants towards the expenses of this investigation, and to H. G. Carson, F.I.M.L.T. for excellent technical assistance.

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ANTAGONISM BETWEEN THE ACTION OF STAPHYLOCOCCAL TOXIN AND THE VENOM OF CERTAIN SNAKES AND INSECTS IN MICE

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Received for publication October 27, 1959

STAPHYLOCOCCAL α toxin which is a better immunizing agent than toxoid (Fisher, 1957) was used in the hyperimmunization of horses for the production of therapeutic antitoxin nearly 30 years ago (Panton, Valentine and Dix, 1931). As one of the characteristic effects of this toxin however is tissue necrosis it has not been used as an immunizing agent in humans.

Early work on the mode of action of staphylococcal toxin showed that it constricts the pulmonary and coronary blood vessels of rabbits (Kellaway, Burnet and Williams, 1930; Burnet, Kellaway and Williams, 1932). More recently Thal and Egner (1954) and Thal (1955) have suggested that vascular spasm and alterations in capillary permeability are responsible for staphylococcal renal cortical necrosis and dermonecrosis in rabbits. There is evidence in mice also that vascular spasm may be a major factor in the production of lesions due to staphylococcal toxin (North and Doery, 1958*a*).

The possibility of finding an antagonist amongst vasodilator drugs to the action of α toxin which would not at the same time affect the antigenicity of the toxin was therefore considered. Among substances tested for such antagonism was a fraction of the venom of the tiger snake (*Notechis scutatus*) rich in haemolysin and poor in neurotoxin (Doery, 1958).

When this fraction (A-1) was mixed with staphylococcal toxin and injected subcutaneously into mice in appropriate amounts a diminution of the dermonecrotic action of the toxin was observed. Protection against this dermonecrotic action of staphylococcal toxin was also noted when the toxin and the snake venom fraction were injected at separate sites.

The results of subsequent observations on the antagonism between the action of this fraction and that of staphylococcal toxin in mice and of other studies which followed, are described below. Many of them have already been reported briefly (North and Doery, 1958*a* and *b*).

MATERIALS AND METHODS

Venoms

Dried crude venoms of the black snake (*Pseudechis porphyriacus*) the king brown snake (*Pseudechis australis*) and the tiger snake (*Notechis scutatus*) were purchased from E. Worrell, Umina, N.S.W. They were dried as soon as possible after collection.

Dried crude venoms of the moccasin snake (*Agkistrodon piscivoris*) and the Indian cobra (*Naja nigricollis*) were obtained from Ross Allen's Reptile Institute, Florida, U.S.A. and the Medical Research Institute, Johannesburg, respectively.

The venom of the honey bee (*Apis mellifera*) for preliminary experiments was used as a simple saline extract of the crude bee stings. For later studies the bee stings were removed,

extracted with saline and an acetic acid-acetone precipitate prepared. The water soluble portion of this precipitate was freeze dried.

Fractionation of the crude venom of the tiger snake (Notechis scutatus)

Three main fractions were available for study (Doery, 1958). Fraction A-1 rich in the haemolysin which is known to be the enzyme phosphatidase A (Doery, unpublished), and fraction A-2, rich in neurotoxin, were separated by chromatography on the resin IRC-50 from a portion of the venom insoluble in 30 per cent methanol at −5°. Fraction B contained a group of methanol soluble neurotoxins which were free of significant phosphatidase A activity.

Estimation of phosphatidase A.—The method used was essentially that of Neumann and Habermann (1955). The venom sample was incubated with an egg yolk suspension and the haemolytic activity of the products of this reaction was determined. A standard preparation of the venom of the black snake served as a control for all estimations.

Detoxification of fraction A-1 of tiger snake venom with formalin

Formalin (0·01 per cent), was added to fraction A-1 (1 mg./ml.). After standing at 37° for 6 days a further 0·01 per cent of formalin was added. After another 6 days 0·1 per cent of formalin was added. After a further 6 days the formalin treated fraction A-1 was removed from the incubator and stored at 2°. At intervals during detoxification the material was sampled and tested for toxicity. The LD50 of the "detoxified" fraction A-1 at the end of the 18 days was 0·135 mg. as compared with 0·005 mg. for the original venom fraction. The detoxified fraction is referred to as toxoid.

Staphylococcal toxin

Two preparations were used. One was prepared from the supernatants of cultures of selected strains grown in Dolman's (1932) medium in an atmosphere of oxygen containing 20 per cent CO_2. After separation, the supernatants were pooled and 0·02 per cent thiomersal added as a preservative. The other toxin was prepared from cultures grown under slightly different conditions. Both supernatants were rich in α-toxin and are referred to simply as staphylococcal toxin.

The LD50 (intravenously) of the former preparation was 1 ml. of 1 : 72 dilution and of the latter 1 ml. of 1 : 60 dilution.

α-Haemolysin titrations.—These were carried out by the method of Burnet (1931).

Experimental animals

White mice were used throughout. For estimating the toxicity (LD50) of venoms and fractions of venoms, mice weighed individually (22–25 g.) were used.

For protection tests mice weighing 20–25 g. were used. In any one experiment mice of approximately equal weight were chosen.

Administration of crude venoms, venom fractions and staphyloccal toxin.—All injections, unless stated otherwise, were given intravenously.

In protection tests the volume of each injection was 0·5 ml.

In toxicity tests the volume of each injection was 1·0 ml.

Toxicity (LD50) tests of crude venoms, venom fractions and staphylococcal toxin.—Groups, each of 4 mice, were injected i.v. with graded doses (50 per cent increments) within the appropriate range.

Challenge of mice with living staphylococci.—Mice were injected intraperitoneally by a modification (North, 1958) of Boake's (1956) method, the staphylococci being suspended in Boake's "clotting mixture" immediately before injection.

RESULTS

Intravenous injection of crude venoms or venom fractions and staphylococcal toxin in mice.

The effect of fraction A-1 of tiger snake venom on the lethal action of staphylococcal toxin.—Following the observation that antagonism exists between the

action of fraction A-1 and the dermonecrotic effect of staphylococcal toxin in mice (North and Doery 1958*a*) the possibility of protecting animals against the lethal action of the toxin was explored.

TABLE I.—*Protection Afforded by LD50/5 Dose of Fraction A-1 of the Venom of the Tiger Snake* (Notechis scutatus) *against Lethal Effect of Staphylococcal Toxin.*

Test No.	Mice surviving 7 days after I.V. injection with: Staph. toxin alone	Staph. toxin and fraction A-1
1	6/15	15/15
2	1/20	15/18
3	2/15	15/15
4	5/20	17/20

Fraction A-1 is itself highly lethal to mice given intravenously. However when the dose of the venom fraction was kept between LD50/4 and LD50/8 protection could be obtained regularly against challenge with a dose of staphylococcal toxin that killed from 50–100 per cent of untreated animals. Table I shows the results of 4 representative experiments in which an LD50/5 dose of fraction A-1 was mixed with staphylococcal toxin and injected intravenously into mice. It will be noticed that in each of these experiments a statistically highly significant degree of protection was afforded against a challenge that killed up to 95 per cent of control animals.

It was found that the length of time that mixtures of venom fraction and toxin were allowed to stand before injection made no difference to the results. Further, it was noted that protection could be demonstrated if fraction A-1 and staphylococcal toxin were injected separately. Table II shows the results of two early

TABLE II.—*Effect of Time Sequence of giving Fraction A-1 in Relation to Challenge with Staphylococcal Toxin.*

Test No.	Mice surviving 7 days after I.V. injection with: Staph. toxin alone	Staph. toxin and fraction A-1	Time of administering fraction A-1
1	9/18	17/18	With staph. toxin.
		18/18	2 min. before staph. toxin.
		12/18	2 min. after staph. toxin.
2	0/12	7/12	With staph. toxin.
		8/12	10 min. before staph. toxin.

Dose of fraction A-1 was LD50/5 (0·002 mg.).

experiments. Test No. 1 demonstrates the fact that protection is good if the venom fraction is given shortly before, but is poor if injected even 2 min. after the staphylococcal toxin. The 2nd test shows that against a certainly lethal challenge, fraction A-1 injected 10 min. beforehand protects at least as well as if it were mixed and injected together with the staphylococcal toxin. As no difference was found in the protective effect whether fraction A-1 was injected

either with, or shortly before the staphylococcal toxin, in subsequent experiments the venom fraction and the toxin were injected together immediately after mixing as a standard procedure.

Fraction A-1 proved to be relatively stable to heat. Immersing a solution in a thin glass test tube in boiling water for 5 min. reduced both its toxicity and its protective power by approximately half. Table III shows the results of three

TABLE III.—*Comparison of Protective Value of Heated and Unheated Fraction A-1 against Lethal Effect of Staphylococcal Toxin.*

Test No.	Dose of fraction A-1 (mg.)	Mice surviving 7 days after I.V. injection with: Staph. toxin alone	Staph. toxin and heated fraction	Staph. toxin and unheated fraction A-1
1	0·002	2/20	17/20	17/20
2	0·002	2/15	13/15	15/15
3	0·002	1/15	15/15	14/15
	0·00066	—	5/15	11/15
	0·00022	—	3/15	4/15

LD50 of unheated fraction A-1 = 0·01 mg.
LD50 of heated fraction A-1 = 0·02 mg.
For each test a different sample of fraction A-1 was heated in a boiling water bath for 5 min.

tests carried out with three different samples heated in this way, in comparison with unheated fraction A-1. It will be noticed that a test (No. 3) in which graded doses of heated and unheated venom fraction were administered was required to show clearly the loss of protective power on heating.

The action of formalin reduced the toxicity of fraction A-1 to a much greater extent (LD50 before toxoiding was 0·005 mg. and afterwards 0·135 mg.) than its protective effect against the lethal action of staphylococcal toxin. Table IV

TABLE IV.—*Comparison of Protective Value of Fraction A-1 before and after Toxoiding against Lethal Effect of Staphylococcal Toxin.*

Sample used for treatment	LD50 (mg.)	Dose in terms of: Mg.	LD50	Mice surviving 7 days after I.V. challenge with staph. toxin: Treated	Untreated controls
Fraction A-1	0·005	0·001	1/5	12/13	1/13
Toxoid	0·135	0·003	1/45	11/12	—

shows the results of a representative experiment in which the protective effect of toxoided and untoxoided fraction A-1 was compared. It will be seen that LD50/45 (0·003 mg.) of toxoided fraction A-1 was as effective in protecting mice as was LD50/5 (0·001 mg.) of untoxoided material. The relative loss of toxicity on toxoiding allowed much more effective dosage with fraction A-1 to be given. Attempts to protect mice against the lethal action of staphylococcal toxin with untoxoided fraction A-1 injected after the toxin gave equivocal results. When mice are injected intravenously with even as little as one LD50 of staphylococcal toxin an occasional mouse dies within a few minutes of challenge. It is obvious

therefore that any protective agent given subsequent to the toxin can only be effective if the challenge is not a highly lethal one that noticeably affects many of the mice within an hour or so of injection. However, as shown in Table V,

TABLE V.—*Protective Effect of Toxoided Fraction A-1 when Administered with and after challenge with Staphylococcal Toxin.*

Treatment with toxoided fraction A-1		Mice surviving 7 days after I.V. challenge with staph. toxin	
Dose of toxoid (mg.)	Time administered	Treated with toxoid	Untreated controls
0·005	With staph. toxin	11/12	5/12
0·005	30 min. after toxin	7/12	—
0·01	30min. after toxin	10/12	—

LD50 of toxoid = 0·135 mg.

statistically significant protection was afforded by an LD50/13·5 dose of toxoided fraction A-1 when administered as long as 30 min. after the injection of the toxin.

The effect of fraction A-1 compared with that of crude tiger snake venom and of fraction A-2 and fraction B.—Given either mixed with staphylococcal toxin or separately beforehand, fraction A-1 regularly protected against the lethal action of the toxin. On the other hand crude tiger snake venom, fraction A-2 and fraction B afforded little or no protection whatever. Table VI shows 3 representa-

TABLE VI.—*Effect of Crude Venom of the Tiger Snake and of Fractions A-2 and B Compared with Effect of Fraction A-1 against Action of Staphylococcal Toxin.*

Test No.	Venom or venom fraction used in treatment	Dose in terms of		Mice surviving 7 days after I.V. challenge with staph. toxin	
		Mg.	LD50	Treated	Untreated controls
1	Fraction A-1	0·002	1/5	14/15	5/15
	Crude tiger snake	0·00015	1/32	3/15	—
	do.	0·0003	1/16	2/15	—
	do.	0·0006	1/8	1/15	—
	do.	0·0012	1/4	0/8	—
2	Fraction A-1	0·001	1/5	12/12	0/12
	Fraction A-2	0·0006	1/5	1/12	—
3	Fraction A-1	0·001	1/4	11/12	0/12
	Fraction B	0·001	1/4	1/12	—
	do.	0·002	1/2	1/12	—

LD50 of fraction A-1 used in tests 1, 2 and 3 was 0·01, 0·005 and 0·004 mg. respectively.

tive experiments in which the action of fraction A-1 was compared with that of crude tiger snake venom fraction A-2 and fraction B. Different lots of fraction A-1 were used in each of the 3 experiments, the LD50 varying between 0·01–0·004 mg. However, the protective action of each lot was essentially the same.

Whilst fraction A-1 was rich in haemolysin and known to contain the enzyme phosphatidase A, fraction A-2 and fraction B consisted mainly of neurotoxins and showed little phosphatidase A activity. Crude tiger snake venom contained relatively less phosphatidase A than did fraction A-1.

The effect of other crude venoms compared with that of the fraction A-1 of the tiger snake.—The activity of other venoms known to contain phosphatidase A was compared with that of fraction A-1 against the lethal effect of staphylococcal toxin. The results of representative experiments are shown in Table VII. It

TABLE VII.—*Comparison of Protective Effect of Fraction A-1 of Tiger Snake Venom with that of Other Crude Venoms against Action of Staphylococcal Toxin.*

Test	Crude Venom	Dose of crude venom in terms of Wt. (mg.)	Dose of crude venom in terms of LD50	Mice surviving 7 days after I.V. challenge with staph. toxin: Treated with crude venom	Treated with fraction A-1	Untreated controls
1	Black snake	0·003	1/4·5	12/12	11/12	2/12
2	King brown snake	0·0015	1/4	14/15	14/15	4/15
3	Indian cobra	0·005	1/8	12/12	7/12	1/12
4	Moccasin snake	0·02	1/5	7/12	12/12	0/12
5	Honey bee	0·005	1/12	20/20	18/20	0/20

Black snake (*Pseudechis porphyriacus*), King brown snake (*Pseudechis australis*), Indian cobra (*Naja nigricollis*), Mocassin snake (*Agkistrodon piscivorus*) and Honey bee (*Apis mellifera*).
Dose of fraction A-1 of tiger snake venom in all tests was LD50/5.

should be noted that the venom which gave least protection, that of the mocassin snake, also showed the lowest phosphatidase A activity.

Crude black snake venom was found to have a high proportion of phosphatidase A in relation to its toxicity. For this reason, and because it was readily available, black snake venom has been largely used in subsequent studies.

Like fraction A-1 of tiger snake venom, it was found to be effective when injected either with or prior to staphylococcal toxin. Table VIII shows

TABLE VIII.—*Protective Effect of Crude Venom of the Black Snake* (Pseudechis porphyriacus) *Administered prior to I.V. Challenge with Staphylococcal Toxin.*

Treatment	Time of death†	Mice surviving 7 days after I.V. challenge
Nil—staph. toxin only	h, h, h, h, h, h, h, h, h, h, h, h	0/12
BSV* with staph. toxin	h, h, 1, 1, 2	7/12
15 min. before toxin	h, h, h, 1, 3, 3, 6	4/11
35 ,, ,, ,,	h, h, h, 1, 1, 3	6/12
60 ,, ,, ,,	h, h, h, h, h, h, h, h, 3	3/12

* The dose of BSV (Black snake venom) was 0·005 mg. (LD50/5) throughout.
† The letter " h " indicates death within 4 hr. and the numerals the day of death after challenge.

the results of an experiment in which groups of mice were given an LD50/5 of black snake venom either mixed with toxin or separately. The challenge dose of staphylococcal toxin in this test was such that all the control animals died within 4 hours of receiving it. However, the protection afforded mice to which black snake venom was given 35 min before challenge, was comparable with that afforded the group to which the venom and staphylococcal toxin were injected together. Some degree of protection was evident in the group challenged 1 hr. before administration of venom.

The protective effect of venoms containing phosphatidase A against challenge with living staphylococci in mice

It is generally accepted that early deaths following experimental staphylococcal infection in animals result from the production and liberation of toxin. Any agent therefore that is antagonistic to the action of staphylococcal toxin might be expected to give some protection against staphylococcal infections.

TABLE IX.—*Protective Effect of Toxoided Fraction A-1 of Venom of Tiger Snake and of Venom of Honey Bee against Intraperitoneal Challenge in Mice with Living Virulent Staphylococci.*

Test No.	Protective agent: Dose and nature of agent used	When injected in relation to challenge	Route	Day of death	Mice surviving 7 days after intraperitoneal challenge with living staphylococci
1	Nil (controls)	—	—	1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1	4/20
	0·02 mg. toxoid	75 min. before	Subcut.	1, 1, 1, 2, 3, 4, 5	13/20
		45 „ after	I.V.	1, 1, 1, 1, 1, 1, 1, 1, 1, 3, 3, 7	7/20
2	Nil (controls)	—	—	1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 3	2/15
	0·02 mg. toxoid	95 min. before	Subcut.	1, 2, 2, 4, 4, 5, 5	8/15
	0·25 ml. saline ext. of bee venom	100 „ „	„	1, 1, 1, 1, 2, 4, 4, 7	7/15

The dose of toxoid was approx. LD50/7 and that of the saline ext. of bee venom LD50/8. Mice were challenged I.P. with living staphylococci suspended in Boake's Clotting Mixture.

Table IX shows the results of two experiments in which groups of mice were challenged with living virulent staphylococci by the intraperitoneal route. In both experiments a large dose of toxoided fraction A-1 of tiger snake venom given subcutaneously more than 1 hr. before challenge, afforded a statistically significant degree of protection. Given intravenously 45 min. after the challenge (Test No. 1) it also afforded definite protection. Two days after challenge there were still 10 survivors out of 20 mice compared with 4 of the 20 control animals. In the only experiment (Test No. 2) carried out with bee venom a simple saline extract administered subcutaneously 100 min. prior to challenge afforded a substantial degree of protection.

Effect of venom on the intraperitoneal (I.P.) or subcutaneous (subcut.) injection of lethal doses of staphylococcal toxin in mice

In most experiments involving I.P challenge fraction A-1 of tiger snake venom was used. The venom fraction given I.P. either before or together with a lethal dose of toxin had no protective effect on the action of the toxin. In some experiments the treated mice actually appeared to be adversely affected as compared with those injected with toxin only. Similarly no protective effect was noted when the venom fraction was given I.V. and the toxin I.P.

In one experiment in which graded amounts of fraction A-1 were injected subcut. together with a lethal dose of toxin, the treated mice were protected to a

highly significant degree as compared with the animals injected with toxin alone. Equivocal results, however, were obtained in other similar experiments when toxin and fraction A-1 were administered subcut. Crude black snake venom injected either subcut. or I.V. likewise failed to protect against a lethal subcut. challenge with toxin.

In vitro *tests with staphylococcal toxin and fraction A-1 of tiger snake venom*

Titration of the staphylococcal toxin for α haemolysin was carried out in parallel with and without the addition of fraction A-1 at a concentration of 0·02 mg./ml. in each tube. In each of 3 tests made on separate days lysis was inhibited slightly but to a definite degree by the venom fraction. Fraction A-1 is itself definitely but weakly haemolytic under these conditions. A test run concurrently with the first of the 3 titrations of staphylococcal toxin cited above, showed that at 0·66 mg./ml. 95 per cent of the red cells of the rabbit were lysed. At 0·12 mg./ml. no lysis was evident.

DISCUSSION

The work recorded above was commenced in an attempt to find a physiological antagonist to the action of staphylococcal toxin (North and Doery, 1958*a*). The attempt was based on the concept that vasoconstriction is the basic factor in the production of lesions caused by staphylococcal toxin and the fact that certain venoms and fractions of venoms increase capillary permeability when injected into laboratory animals.

Whilst fractions A-1, A-2 and the crude venom of the tiger snake itself and to a lesser extent fraction B all increase capillary permeability, only fraction A-1 was found to protect mice against the lethal action of staphylococcal toxin. It would appear then that the protective action of fraction A-1 is not due to its action as a vasodilator *per se*. Fraction A-1 however has a much higher content of the enzyme phosphatidase A in relation to its lethal effect than have either of the other fractions or the crude venom.

Further, it soon became evident that all the venoms or fractions of venom found to protect mice against the action of staphylococcal toxin had a relatively high content of phosphatidase A. The role that this enzyme plays in defence against the action of bacterial toxins is dealt with separately (Doery and North, 1960). Its action on the antigenicity of staphylococcal toxin will be the subject of a later report.

It seems pertinent, however, at this stage to call attention to certain features concerning the protective action of these venoms. Whilst their protective effect can be regularly shown against perhaps 2 or even more LD50's given intravenously the protection is by no means complete. Further, the evidence to date suggests that the venom does not act directly on the toxin itself. The protective action when venom and toxin are mixed, and allowed to stand for 30 min. or more before injection, is no greater than when the 2 are mixed immediately before injecting them, or when the toxin is injected separately shortly before the toxin.

The first indication of antagonism between the action of venom and that of staphylococcal toxin was obtained when they were both injected subcutaneously with lessening of the dermonecrotic action of the toxin. Nevertheless protection by venom against the lethal action of staphylococcal toxin injected subcutaneously

has been found much more difficult to demonstrate than protection against its lethal action intravenously.

The lethal effect of staphylococcal toxin when injected intraperitoneally or intravenously is very similar. Yet venoms effective against the lethal action of toxin given intravenously do not protect against the intraperitoneal injection of toxin. This finding is at variance with the undoubted benefit shown by venom in the treatment of intraperitoneal injections with virulent living staphylococci.

All the venoms used are highly lethal to mice and little is known yet how to use them to the best advantage. It is, therefore, perhaps more remarkable that certain venoms can be shown to protect animals against the lethal effect of toxin by the intravenous route than is the fact that they have failed to protect under other experimental conditions. A possible reason for these apparent anomalies will be offered in the paper following (Doery and North, 1960).

SUMMARY

The present study started with the observation that antagonism exists between the dermonecrotic effect of staphylococcal toxin and the action of a preparation of tiger snake venom. The venom protects whether given subcutaneously to mice with the toxin or separately. This venom preparation and others also containing phosphatidase A given intravenously protect mice against the lethal effect of toxin by the same route. Venom preparations with little phosphatidase A activity have failed to protect.

Venom preparations effective against intravenous challenge with toxin are relatively ineffective against a lethal subcutaneous challenge and apparently quite useless against intraperitoneal injection of toxin. By contrast, they protect to a limited extent against intraperitoneal challenge with living staphylococci.

The active principle in these venoms is relatively heat stable and its effectiveness is increased by toxoiding with formalin.

We are greatly indebted to Ross Allen's Institute, Florida, U.S.A. and to the Director, Medical Research Institute, Johannesburg, for gifts of venom ; also to Mr. G. Pawlyszyn and Miss Edith Russell for able technical assistance.

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FACTORS INVOLVED IN THE ACTION OF VENOMS AGAINST BACTERIAL TOXINS IN EXPERIMENTAL ANIMALS

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Received for publication October 27, 1959

IT has already been shown that a fraction of the venom of the tiger snake (*Notechis scutatus*) and a number of other crude venoms containing the enzyme phosphatidase A protect mice against the lethal effect of staphylococcal toxin (North and Doery 1958a and b, 1960). The fraction of the venom of the tiger snake in which this enzyme was concentrated also had some neurotoxic activity. With a view to studying the action of phosphatidase A in a form as free as possible from other venom components the enzyme was prepared from the venom of the black snake (*Pseudechis porphyriacus*). This venom is less toxic than that of the tiger snake and contains phosphatidase A in high concentration. Two fractions in which this enzyme was concentrated have been studied.

Although the original concept in this project was a physiological one, only the biochemical aspects have so far been studied. The possibility that the products of the action of phosphatidase A on phosphatides in the animal body were responsible for the protective effect was considered. These reaction products are lysophosphatides and long chain fatty acids. The interaction of long chain fatty acids both with living bacteria and bacterial toxins is well known. However, the results of the recorded work in this field are conflicting (Nieman 1954). An attempt has been made to define the conditions under which certain long chain fatty acids interact with bacterial toxins.

The results of these studies with phosphatidase A and the products of its reaction with lecithin form the basis of this communication.

MATERIALS AND METHODS

Dried crude venom of the black snake (*BSV*).—This has already been described (North and Doery 1960).

Estimation of phosphatidase A.—This has already been described (North and Doery 1960).

Separation of phosphatidase A from BSV.—BSV was fractionated by procedures similar to those used for the fractionation of the venom of the tiger snake (Doery 1958), briefly, adsorption on barium carbonate and chromatography on the amberlite resin IRC—50 at pH 7·5 and 6·0 (Fig. 1).

Lecithins.—(a) L-α-(dimyristoyl) lecithin synthesized by Prof. E. Baer, Toronto, Canada. Subsequent supplies were obtained from La Motte Chemical Products Co., U.S.A. (b) Egg Lecithin. Lecithin was extracted from egg yolk and freed from ninhydrin reacting material by adsorption on alumina (Lea, Rhodes and Stoll, 1955).

Lysolecithins (myristoyl and egg).—These were prepared from L-α-(dimyristoyl) lecithin and egg lecithin, respectively, using fraction X–2 of BSV as the source of phosphatidase A, by the method of Hanahan, Rodbell and Turner (1954), and recrystallized from warm ethanol to remove any adsorbed enzyme (Saunders 1957).

Sodium salts of fatty acids.—Fatty acids were dispersed in 0·1N NaOH with warming, then diluted with saline to the appropriate concentration and the pH adjusted to 7·3–7·5.

Ethyl oleate.—A commercially pure preparation was dispersed in saline in a Waring blender with the aid of Tween 80.

Haemolysis of whole blood and washed red cells obtained from heparinized animals (rabbit and mouse) by lysolecithin.—The method was essentially that of Neumann and Habermann (1954). All dilutions were made with Sørenson's buffer pH 7·0 diluted 1/5 with saline.

Staphylococcal toxin.—This has already been described (North and Doery 1960).

Diphtheria and tetanus toxins.—These were obtained from the Production Division of these Laboratories.

Experimental animals.—White mice 20–25 g. weight and Guinea pigs, 230–270 g. weight, were used.

Administration of BSV fractions, crude BSV, bacterial exotoxins, fatty acid compounds and lysolecithins, to mice.—Injections were given i.v. as already described (North and Doery 1960).

Administration of diphtheria toxin and sodium oleate to guinea pigs.—Diphtheria toxin alone or mixed with sodium oleate was injected subcutaneously.

Toxicity (LD 50) tests in mice.—These were carried out as already described for venom preparations and staphylococcal toxin (North and Doery 1960).

RESULTS

The procedures adopted for the separation of fractions of the venom of the black snake containing phosphatidase A are outlined in Fig. I. Phosphatidase

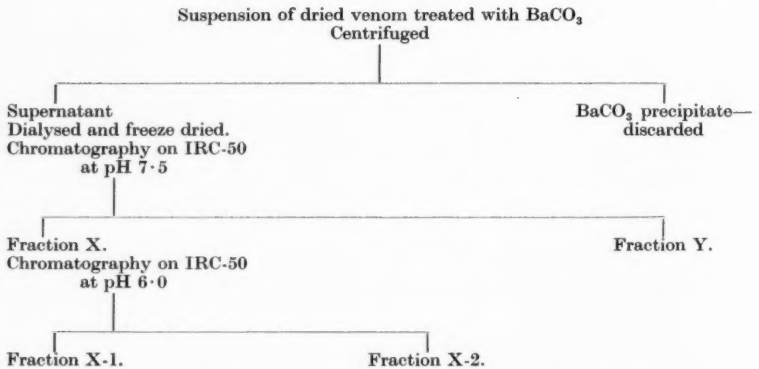


FIG. 1.—Fractionation procedure for the venom of the Black snake.

A activity was found to be associated with fractions X-1, X-2 and Y, and so far only fractions X-1 and X-2 have been studied in detail. The properties of these 2 fractions differed from one another as judged by chromatography, paper electrophoresis, stability on dialysis and relative concentration of phosphatidase A. The phosphatidase A activities of fraction X-1 and fraction X-2 relative to that of the crude venom were 0·5 and 2 respectively. Both protected mice against the lethal effect of staphylococcal toxin when mixed with the toxin and injected intravenously, the degree of the protection being proportional to the relative phosphatidase A activity of each fraction (Table I). In addition, highly significant protection was provided by injecting fraction X-2, 15 min. prior to challenge with staphylococcal toxin (Table II).

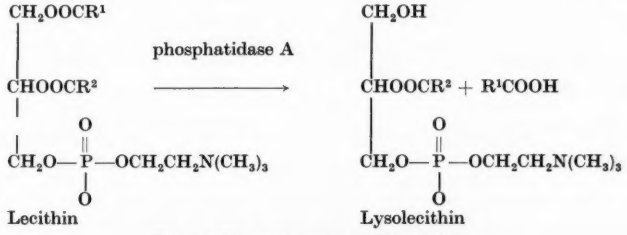
TABLE I.—*Protection Afforded by the Venom of the Black Snake* (Pseudechis porphyriacus) (*BSV*) *and Fractions Containing Phosphatidase A, Against the Lethal Effect of Staphylococcal Toxin.*

Material injected	Phosphatidase A activity of fraction relative to BSV	Dose of venom (mg.)	Mice surviving i.v. challenge 7 days
Staph. toxin alone	—	—	3/10
,, ,, + BSV	—	0·004	7/10
,, ,, + fract. X-1	0·5	0·0063	9/10
,, ,, ,, ,,	0·5	0·0016	3/10
,, ,, + fract. X-2	2·0	0·0016	9/10
,, ,, ,, ,,	2·0	0·0004	3/10

TABLE II.—*Protective Effect of Fraction X-2 of BSV when Administered with or prior to Challenge with Staphylococcal Toxin.*

Material injected	Dose of fraction X-2 (mg.)	Mice surviving i.v. challenge 7 days
Staph. toxin alone	—	3/12
,, ,, + fract. X-2	0·0025	12/12
Fract. X-2 given 15 min. prior to staph. toxin	0·0025	11/12

The products of the action of phosphatidase A (Fig. 2) on a particular phosphatide, namely lecithin were then considered in turn. Table III summarizes the results of experiments designed to explore the part, if any, of lysolecithins, in



Lecithin Lysolecithin

R^1 and R^2 are long chain fatty acids.

FIG. 2.—Action of phosphatidase A on lecithin.

the protective mechanism being studied. Two lysolecithins, namely myristoyl, prepared from a synthetic lecithin containing 2 saturated fatty acids, and egg lysolecithin, from a lecithin known to contain a mixture of saturated and unsaturated fatty acids, were examined for possible protective effect against the lethal action of staphylococcal toxin. The results of these tests show that both lysolecithins protect mice against the lethal effect of the toxin, either when injected intravenously, together with the toxin, or 10–15 min. prior to challenge.

Concerning the part played by long chain fatty acids in the protective mechanism being studied, the effect of sodium oleate on staphylococcal toxin was studied in some detail.

It was found that when sodium oleate and toxin were injected intravenously into mice immediately after mixing, the activity of the toxin was definitely

TABLE III.—*Protective Effect of BSV and Lysolecithins when Administered with or prior to Challenge with Staphylococcal Toxin.*

Expt. No.	Material injected	Dose of protective agent (mg.)	Mice surviving i.v. challenge 7 days
1	Staph. toxin alone	—	1/12
	,, ,, + BSV	0·004	12/12
	,, ,, + Lysolecithin (myristoyl)	0·05	8/12
	Lysolecithin (myristoyl) given 10 min. prior to staph. toxin	0·05	5/12
2	Staph. toxin alone	—	1/12
	Lysolecithin (myristoyl) given 10 min. prior to staph. toxin	0·2	6/12
3	Staph. toxin alone	—	1/12
	,, ,, + lysolecithin (egg)	0·4	13/13
	Lysolecithin (egg) given 15 min. prior to staph. toxin	0·4	8/12

Lethal dose of both lysolecithins = 2–3 mg.

enhanced. When sodium oleate was injected immediately before or after the challenge with staphylococcal toxin, it failed to protect the mice. By contrast, complete inactivation of a certainly lethal dose of toxin was regularly obtained, by incubation for 30 min. at 37° with 0·75 mg. of sodium oleate. The results of a representative test are shown in Table IV. Diphtheria toxin and tetanus toxin were inactivated under similar conditions (Table IV).

TABLE IV.—*Inactivation of Bacterial Toxins by Sodium Oleate at* 37°.

Material injected	Sodium oleate (mg.)	Experimental animal	Route	Survivors at 7 days
Staph. toxin alone	—	Mouse	i.v.	0/15
,, ,, + sod. oleate	0·75	,,	,,	15/15
Diphtheria toxin alone	—	Guinea-pig	s.c.	0/6
,, ,, + sod. oleate	10	,,	,,	6/6
Tetanus toxin alone	—	Mouse	,,	0/15
,, ,, + sod. oleate	0·75	,,	,,	15/15

Sodium linoleate also completely inactivated staphylococcal toxin. However, the sodium salt of the saturated fatty acids tested, stearic, myristic, palmitic, and caprylic, and the ethyl ester of oleic acid failed to inactivate staphylococcal toxin. Table V summarizes the results of a series of mouse protection tests. In each experiment, one group of mice was tested with a mixture of the staphylococcal

TABLE V.—*Relative Effectiveness of Compounds of Fatty Acids as Inactivators of Staphylococcal Toxin at* 37°.

Fatty acid compound	Minimum amount to inactivate (mg.)	Lethal dose i.v. of test substance (mg.)
Sodium oleate	0·025	>3
Sodium linoleate	0·075	>3
Sodium stearate	No inactivation	0·5–1
Sodium palmitate	,, ,,	0·5
Sodium myristate	,, ,,	0·25
Sodium caprylate	,, ,,	>3
Ethyl oleate	,, ,,	>10

toxin and 0·025 mg. of sodium oleate, as a positive control. In no instance did this dose of sodium oleate fail to give complete protection.

The affinity of albumin for fatty acids, which is well established, is demonstrated by the results shown in Table VI. A mixture containing a final concentra-

TABLE VI.—*Effect of Albumin* (1·5 *per cent*) *on the Inactivation of Staphylococcal Toxin by Sodium Oleate* (0·15 *per cent*) *at* 37° *for* 30 *min.*

Material injected (I.V.)	Mice surviving challenge 7 days
Staph. toxin alone . . .	1/5
,, ,, + albumin . .	0/5
,, ,, + sod. oleate .	5/5
,, ,, + sod. oleate + albumin	0/5

tion of 1·5 per cent albumin and 0·15 per cent sodium oleate failed to inactivate in 30 min. at 37° a dose of staphylococcal toxin which was completely inactivated by the same amount of sodium oleate alone. Thus the fatty acid, when mixed with albumin and staphylococcal toxin, was preferentially adsorbed by the albumin. This explains the difficulty found in protecting mice when the toxin and fatty acid are injected separately. Significant protection was sometimes obtained by injection of the largest amount of oleate the mice would tolerate 30 min. or more before the challenge with toxin. The result of one test in which protection was obtained is shown in Table VII. However, protection was not consistently obtained.

TABLE VII.—*Protective Effect of Sodium Oleate Administered 18 hr. prior to i.v. Challenge with Staphylococcal Toxin.*

Material injected	Time of death*	Mice surviving 7 days after i.v. challenge
Staph. toxin alone . . .	h, h, h, h, 1, 2, 2, 2, 3, 4, 4, 5 .	0/12
3 mg. sod. oleate 18 hr. prior to staph. toxin	1, 2, 2, 2, 3, 7 .	6/12

* The letter " h " indicates death within 6 hr. and the numerals the day of death after challenge.

The results of the determination of the haemolytic activity of myristoyl lysolecithin on the red cells of the rabbit and mouse, prepared from blood of heparinized animals are shown in Table VIII. No significant difference was shown when the tests were carried out with either whole blood or washed red cells. Results of the same order were obtained for the haemolysis of red cells from citrated human blood, by both egg and myristoyl lysolecithins. Haemolysis was actually almost instantaneous, little, if any, additional lysis occurring on incubation.

DISCUSSION

Having established a direct relationship between the concentration of phosphatidase A in a venom fraction and its protective activity, it was of interest to examine the possible mechanisms involved. It would now appear that both products of the action of this enzyme on phosphatides, namely, lysophosphatides and long chain fatty acids, are involved.

TABLE VIII.—*Haemolytic Activity of Lysolecithin (myristoyl) on 4 per cent Whole Blood and 2 per cent Washed Red Cells of Rabbit and Mouse Blood Collected after Injection of Heparin.*

Lysolecithin (mg.)	Per cent haemolysis			
	Rabbit		Mouse	
	Whole blood	Red cell suspension	Whole blood	Red cell suspension
0·25	100	100	75	100
0·125	100	100	50	75
0·062	35	100	25	40
0·031	5	50	tr	17
0·016	—	12	—	tr

The lysophosphatides which were studied contained entirely (myristoyl lysolecithin) or almost entirely (egg lysolecithin, Rhodes and Lea, 1956) saturated fatty acids. Other types remain to be examined.

While it has been known for many years that lysophosphatides exist in animal tissues (Belfanti, 1924), recent work identifying them in blood (Phillips, 1957; Phillips and Roome, 1959) and normal tissues, (Hadju, Weiss and Titus, 1957; Marinetti, Witter and Stotz, 1957) reflects the increasing interest in the function of these compounds.

Phosphatidase A is found in the pancreas (Hanahan, 1954; Rimon and Shapiro, 1959) while lysolecithin itself is a substrate for enzymes located in the pancreas (Shapiro, 1953), liver (Dawson, 1956) and intestinal mucosa (Epstein and Shapiro, 1959), with fatty acid and glycerophosphorylcholine as reaction products.

Recently, Neumann and Habermann (1957) have drawn attention to some established properties of lysophosphatides including their solubilizing effects, their capacity to disintegrate cellular structures and alter permeability, all of which may be related to their high surface activity.

Concerning their role in the animal body, Ponder (1948) suggested that the natural turnover of red cells might be due to the action of lysophosphatides. In this connection, the fact that certain combinations of lecithin and lysolecithin mask the haemolytic activity of the latter (Saunders, 1957) suggests the availability of a balanced mechanism *in vivo*. However, enzymes which further degrade lysolecithin no doubt control any accumulation of this compound. A digitalis like action was described by Hadju, Weiss and Titus (1957) for palmitoyl lysolecithin found in normal serum, and more recently Maclagan (1958) attributed an inhibitory effect on blood coagulation to lysophosphatides.

The only information concerning the concentration of lysophosphatides in the animal body has been provided by Phillips (1957) and Phillips and Roome (1959) who found that 7 per cent of the total phosphatides (1·5–2·0 mg./ml.) of human plasma existed as lysolecithin. Assuming a similar level in the mouse, the total content of lysolecithin in the plasma of a 20 g. mouse would be 0·2–0·3 mg. In the protection tests reported above, amounts of lysolecithin of this order injected intravenously, were required to protect mice against a lethal challenge by staphylococcal toxin.

Considering the other product of the action of phosphatidase A, the results reported above demonstrate the known interaction of long chain fatty acids and

proteins. It is known that long chain fatty acids do not normally exist free in the blood stream but are largely bound to serum albumin. Goodman and Shafrir (1959) found that the normal level of unesterified fatty acids in fasting human plasma was 5×10^{-4} moles/litre or just less than 1 mole of fatty acid per mole of albumin. Furthermore, Gordon (1955) found that on adding sodium oleate to normal serum in an ice bath it combined with albumin until a ratio of approximately 3 moles per mole of albumin was reached. However, our experience (Table VI) is that at 37° far more sodium oleate combines with albumin.

It seemed unlikely that sodium oleate injected separately would protect experimental animals against bacterial toxins, when normal serum albumin has such an affinity for fatty acids. However, it has been demonstrated that, under certain conditions, sodium oleate introduced into the circulation may be sufficiently loosely combined to interact with a subsequent challenge of toxin.

Assuming that the level of the unesterified fatty acids in mouse and human plasma are the same, *i.e.* 5×10^{-4} moles/litre or approximately 0·15 mg./ml., it might be expected that the total plasma of a 20 g. mouse would contain 0·3 mg. One tenth of this amount as sodium oleate completely inactivated, at 37° *in vitro*, an amount of toxin which constituted a lethal challenge. By contrast, when the sodium oleate was administered to the mice separately from the toxin a dose equal to ten times the normal plasma content of unesterified fatty acids was required to protect (See Table VII).

When related to normal physiological levels the amounts of fatty acid or lysolecithin required to protect mice are certainly enormous. On the other hand, micro amounts of phosphatidase A give striking protection against the lethal action of staphylococcal toxin. The enzyme is a normal constituent of the animal body and phosphatides are essential components of all cells. In addition to its effect against the action of staphylococcal toxin, a venom fraction also afforded mice some protection against tetanus toxin (North and Doery, 1958b). Further, the non-specific interaction of long chain fatty acids with toxins in general is well known. It is suggested that an observation by Fisher (unpublished) may be relevant to the fact that brain tissue is rich in phosphatides. He found that intracerebral injection of sublethal doses of staphylococci in mice failed to produce macroscopic brain lesions whilst suppurative osteomyelitis of the skull often developed at the site of the needle puncture.

These facts suggest an attractive but highly speculative hypothesis concerning a mechanism of natural protection against the action of noxious agents such as certain toxins. It is suggested that the enzyme is mobilized in some way by the toxin. The enzyme then interacts with phosphatide at the cell surface releasing fatty acid and lysophosphatide. In view of the wide occurrence of several enzymes other than phosphatidase A which release fatty acids from phosphatides, these enzymes may equally well contribute to a natural defence mechanism. An extension of the hypothesis may help to explain individual variation in susceptibility. At present, genetic difference is invoked. It is not difficult to visualize this difference involving varying capacity to utilize (or mobilize) the appropriate enzyme.

Many factors are involved in the difficulty of protecting mice against the lethal effect of staphylococcal toxin by routes other than the i.v. These include variables in the relative distribution of toxin, enzyme and the products of their reaction.

Toxin injected subcutaneously causes local tissue necrosis and vascular damage. The greater the damage, the poorer is the absorption. Because of the poor absorption it takes at least two or three times as much toxin to kill a mouse by the subcutaneous as by the i.v. route. Administration of phosphatidase A by lessening the local vascular damage allows more of the toxin to get into the general circulation and exert an added lethal effect. The application of this reasoning to the protection of mice against lethal challenge subcutaneously will be dealt with in a later paper. The factors involved in the failure of phosphatidase A to protect against i.p. challenge are almost certainly more complex and have not yet been studied.

SUMMARY

The protection of mice against the lethal effect of staphylococcal toxin by fractions of the venom of the black snake is proportional to their concentration of phosphatidase A. The products of the action of this enzyme on phosphatides, namely, lysophosphatides and long chain fatty acids were examined. The two lysolecithins tested, protected mice either when injected together with, or prior to the challenge dose of toxin. Of the fatty acids studied, only unsaturated ones inactivated staphylococcal toxin. Given separately, an unsaturated fatty acid sometimes protected against the action of the toxin. Based on these and other observations it is suggested that phosphatidase A and other enzymes take part in a natural defence mechanism against bacterial toxins.

We are indebted to Professor E. Baer, University of Toronto, for a gift of L-α— (dimyristoyl) lecithin and also Miss Frances Bates for very able technical assistance.

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THE EFFECT OF CORTISONE IN EXPERIMENTAL MOUSE TYPHOID

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Received for publication October 29, 1959

THE administration of cortisone enhances infections caused by a wide variety of microbial pathogens in several species of experimental animals. This effect is readily demonstrated in acute infection with staphylococci (Thomas, 1952), or pneumococci (White and Marshall, 1951), but also occurs in chronic diseases such as brucellosis (Abernathy, 1951) and tuberculosis (Hart and Rees, 1950; Lurie, Zappasodi, Danneberg and Swartz, 1951). Relatively indolent infections caused by organisms which are either not natural pathogens for the animal employed or which are introduced by unnatural routes may also be enhanced by cortisone, *e.g.* rabbit infections caused by *Salmonella typhi*, (Lurie, Zappasodi, Danneberg and Swartz, 1951), streptococci (Mogabgab and Thomas, 1950, 1952), and *Treponema pallidum*, (Turner and Hollander, 1950). Experimental infections with polioviruses (Schwartzman, 1950) or influenza viruses (Kalter, Stuart and Tepperman, 1950) are similarly enhanced, and the outcome of experimental malaria may also be influenced by cortisone (Kass, Geiman, Ingbor, Ley, Harris and Finland, 1951).

There is no evidence of any direct effect of cortisone on the properties of infective agents (Kass and Finland, 1953) and the wide range of host-parasite relationships affected by the hormone suggests an activity against some fundamental aspect of host defence mechanisms. Many of the different biological and pharmacological effects of cortisone have been investigated from this point of view and the extensive literature has been fully reviewed by Thomas (1953, 1954, 1955). Most of the evidence is in favour of the view of Halpern and his colleagues (Halpern, Benacerraf and Biozzi, 1953; Biozzi, Benacerraf and Halpern, 1953; Benacerraf, Halpern, Biozzi and Benos, 1954) that enhancement of infection by cortisone is largely attributable to reticulo-endothelial dysfunction.

Although the introduction of cortisone into an experimental model infection may not help in defining what factors host-resistance depend upon, it can be justified as a means of determining the ability of different strains of a bacterial species to multiply *in vivo* when host reactions are suppressed, and as a method of comparing the degree or stability of host-resistance at different stages in the disease.

Previous investigations in experimental mouse typhoid (Hobson, 1956, 1957a) have shown that survivors of infection with virulent strains of *Salmonella typhimurium* commonly achieve an equilibrium in which moderate numbers of the infecting bacteria persist in the tissues but fail to cause late relapse of overt disease. This stable carrier state has been attributed to host defence factors

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stimulated during infection rather than to any loss of determinants of virulence by the infecting organism (Hobson, 1957a, b, c). In the following experiments the effect of cortisone upon this acquired resistance has been studied.

Strains of *Salm. typhimurium* of low virulence for normal mice probably vary considerably in respects other than their common inability to kill. For example, the strain S2/R previously described (Hobson, 1957b) could establish persistent latent disease in most inoculated animals, whereas the strain M206 described by Maaloe (1948a and b) may be completely eradicated from the tissues within a short time of injection. This failure to establish infection or to continue to a fatal outcome could be due to an increased susceptibility of these strains to host defence mechanisms or to an inherent inability to flourish in the biochemical environment of normal mouse tissues. In the experiments detailed below the behaviour of these strains has been reinvestigated in cortisone-treated animals.

MATERIALS AND METHODS

The methods employed in the experimental model infection have all been described previously (Hobson, 1956 ; 1957*a* ; 1957*b*). Swiss white mice were used at 20 g. weight and segregated immediately after inoculation into individual containers to exclude cross-infection. Two of the strains of *Salm. typhimurium* (S2/446 and the mutant S2/R of reduced virulence) have also been described previously. The third strain, M206 described originally by Maaloe (1948*a* ; 1948*b*), was kindly provided by Dr. D. Rowley. This strain possesses the normal antigenic constitution and biochemical properties of *Salm. typhimurium*.

A sterile saline suspension of cortisone acetate (Boots) was used ; the dosage was based on that described by Hart and Rees (1950). In the chronic carrier experiments where the average weight and the weight-range was greater than in freshly-selected groups, the dosage (equivalent to 25 mg./kg. body weight) was adjusted for each animal. The required dose was given in one daily injection of 0·5 ml. subcutaneously.

RESULTS

Cortisone administration in recently-established infection

Groups of 20 mice were inoculated intraperitoneally with 5000 organisms of strains S2/446, S2/R or M206 ; 24 hr. later, cortisone administration was started in 10 randomly-chosen animals of each infected group and in 20 non-infected mice of similar weight. Twelve daily doses of cortisone were given ; all animals alive at this time were observed until 28 days after inoculation and were then killed, and bacterial counts of each organ were made by methods previously described (Hobson, 1956). All deaths were confirmed bacteriologically.

All the cortisone-treated animals lost weight ; in the non-infected group the average loss over the 12 days was 2 g. One of this group died on the 10th day but no bacterial pathogens were found. The others survived until the 28th day, and no organisms were found in organ cultures when they were killed.

The results in the infected groups are shown in Table I. In the case of the virulent strain S2/446, the mean time to death was reduced in the cortisone-treated group though the time before the first death occurred was no shorter than in the non-treated group.

Infection with the less virulent strain S2/R was strikingly modified. Of the 10 cortisone-treated mice 9 died ; none of the deaths occurred before the 5th day of treatment but all nine before the 12th. All the infected untreated group survived for 28 days and 9 were persistently infected at that time.

TABLE I.—*The Effect of Cortisone* (25 *mg./kg./day for* 12 *days*) *When Treatment is Begun* 24 *hr. After Injection.* (*Total Period of Observation* = 28 *days*)

Bacterial challenge strain		Deaths	Mean time to death (days)	Number of survivors persistently infected
S2/446	Cortisone	10/10	6·2	0/0
	No cortisone	8/10	11·5	2/2
S2/R	Cortisone	9/10	9·7	1/1
	No cortisone	0/10	—	9/10
M206	Cortisone	3/10	17·7	2/7
	No cortisone	0/10	—	4/10

In a control group of 20 non-infected mice treated with cortisone, one died on day 10; no bacterial pathogen was found.

Enhancement of infection with the strain M206 was less well marked. In the untreated group all survived; 6 were free from infection by the 28th day and 4 were carrying small numbers of *Salm. typhimurium*. In the cortisone-treated group, 3 mice died after cortisone was discontinued, 2 survivors were chronic carriers and 5 were free from infection.

The absence of deaths in the first 5 days of infection followed by a rapid increase in mortality thereafter in 2 of the groups receiving cortisone suggested that there might be a lag period before the full biological effect of the drug was exerted. Since most mice which eradicate M206 infection are known to do so within the first 2 days of infection (Hobson, unpublished) and since a detectable increase in host-resistance to *Salm. typhimurium* has occurred as early as the 4th day after infection (Hobson, 1957c) it seemed probable that the maximum effect of cortisone on this host-parasite relationship could be demonstrated only by excluding the possibility of a lag period.

The effect of pre-treatment with cortisone

Cortisone administration was started in 50 normal mice, and 30 similar animals were kept as untreated controls. After 5 days, groups of 10 treated and 10 untreated mice were inoculated intraperitoneally with 5000 organisms of either S2/446, S2/R or M206. Cortisone was continued in the treated mice until the 12th day. The results on the 28th day of infection are shown in Table II.

TABLE II.—*The Effect of Cortisone* (25 *mg./kg./day for* 12 *days*) *When Treatment is Begun* 5 *Days Before Infection.* (*Total Period of Observation* = 28 *days*)

Bacterial challenge strain		Deaths	Mean time to death (days)	Number of survivors persistently infected
S2/446	Cortisone	10/10	2·4	0/0
	No cortisone	10/10	11·2	0/0
S2/R	Cortisone	10/10	5·1	0/0
	No cortisone	3/10	13·3	7/7
M206	Cortisone	7/10	5·0	3/3
	No cortisone	1/10	13·0	1/9

When cortisone treatment was established well before infection, mice succumbed rapidly after inoculation of any of the 3 bacterial strains. However, the relative order of virulence of the strains was preserved to some extent despite the augmenting effect of cortisone. Thus, S2/446 infection killed all the treated mice in 2–3 days, whereas the mean time to death was 5·1 days in S2/R infection, and when infected with M206 three mice survived the whole period as chronic carriers.

In these experiments it was noted that the interval between the first signs of infection (reduced mobility and ruffled fur) and death was much shorter in the cortisone-treated than the untreated groups. This was especially marked in the infection with the strain S2/446 where all the mice were grossly ill 18 hr. after infection and 6 died within the next 12 hr. This picture had been seen previously only when doses of *Salm. typhimurium* of the order of 1×10^6 organisms had been inoculated ; the manifestations then had not been dependent on the titratable virulence of the strain used, and were presumed to represent the toxic effects of the high concentrations of lipopolysaccharide in the bacterial inoculum.

The following experiments were performed to show whether cortisone allowed bacterial multiplication *in vivo* to occur faster than normal, with an earlier attainment of the usual critical toxic bacterial level (Hobson, 1957a), or whether deaths in cortisone-treated mice occurred with smaller final bacterial populations than normal, suggesting increased susceptibility to endotoxin.

The final bacterial population and post-mortem appearances of cortisone-treated infected mice

Ten mice were treated with cortisone as above ; after the 5th dose, these mice and 10 untreated animals were inoculated intraperitoneally with 5×10^3 organisms of the strain S2/446. Mice from each group were killed when they became so grossly ill that death within the next 6 hr. appeared certain ; quantitative bacterial counts were made of the blood and of each organ. There was no significant difference in the degree of bacteraemia or in the relative distribution of infection in the 2 groups. The mean total final bacterial population (the sum of the total counts for each organ and for the blood) was $3{\cdot}5 \times 10^8$ in the cortisone-treated group and $1{\cdot}2 \times 10^8$ in the untreated. On post-mortem examination there were no obvious haemorrhages and no evidence of bilateral cortical necrosis of the kidneys as described by Thomas and Smith (1954) in rabbits injected with meningococcal endotoxin after cortisone administration.

In similar experiments with the strains S2/R and M206 final bacterial populations all fell between $1{\cdot}5 \times 10^8$ and 5×10^8, regardless of the day of infection on which the animal became moribund, or of cortisone treatment.

The fate of Salm. typhimurium *injected intravenously in mice pretreated with cortisone*

Thirty mice were given cortisone daily and 20 similar mice were kept as untreated controls. After 5 days all were injected intravenously with 2×10^5 organisms of the strain S2/446, prepared as in previous experiments (Hobson, 1957b). Daily cortisone injections were continued after infection. Pairs of mice from each group were killed immediately after infection, at hourly intervals for the next 6 hr. and thereafter at 24, 48 and 72 hr. where survivors were avail-

able. Total bacterial counts are shown in Fig. 1 and the blood clearance rates are given in Fig. 2.

In the untreated animals the progress of infection was similar to that previously described (Hobson, 1957b). In the cortisone-treated group, blood-clearance was not significantly impaired and by 6 hr. after infection as many

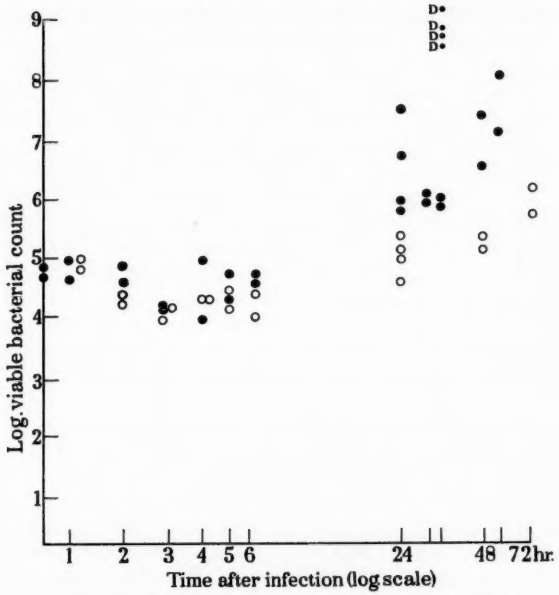

FIG. 1.—The effect of cortisone on the course of infection after intravenous inoculation of 2×10^5 organisms of *Salm. typhimurium* S2/446.

(Points = total bacterial populations.)
● Cortisone-treated mice.
D Deaths in cortisone-treated group
○ Untreated mice.

organisms had been removed from the circulation as in untreated mice. There appeared to be no difference over the first 6 hr. in the numbers of cleared organisms remaining viable in the tissues. Thereafter total bacterial populations rose to higher levels in cortisone-treated than untreated mice, and the degree of bacteraemia was greater. In the overnight period from 32–36 hr. after infection, 4 of the treated mice died. Total bacterial counts in these animals ranged from $7{\cdot}5 \times 10^8$ to $2{\cdot}7 \times 10^9$, but it is obviously uncertain how much of this represents post-mortem bacterial multiplication.

The effect of cortisone in the chronic stable carrier

Since cortisone produced a definite augmentation of recent intraperitoneal infection with 5×10^3 organisms, it was decided to give a similar regime of treatment to chronic stable carriers of the strain S2/446. Adequate numbers of

mice with persistent infection could be obtained by treatment early after inoculation with furazolidone (Hobson, 1956).

Forty mice were inoculated intraperitoneally with 5×10^3 S2/446 organisms and, after segregation as usual into single containers, were given furazolidone by oesophageal catheter in a dosage of 25 mg./kg./day for 8 days. After 28 days infection there were 36 survivors and these were randomly divided into 2 groups, 18 receiving cortisone 25 mg./kg./day for 12 days, and the remainder being left untreated.

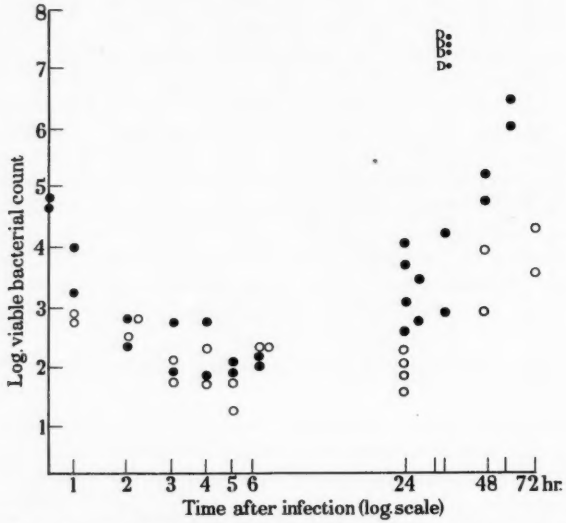

FIG. 2.—The effect of cortisone on blood clearance after intravenous inoculations of 2×10^5 organisms of *Salm. typhimurium* S2/446.
(Points = bacterial count per ml. of blood.)
● Cortisone-treated mice.
D Deaths in cortisone-treated group.
○ Untreated mice.

In the cortisone-treated group, 4 mice died during the period of administration and a profuse growth of *Salm. typhimurium* was obtained from each organ. No illness occurred in any other animal throughout the experiment. The survivors, and members of the untreated group, were killed at intervals over the 10 days following withdrawal of cortisone. All animals in each group were carriers of *Salm. typhimurium*, but in all cases the infection was confined to liver and spleen, and the total bacterial population was less than 1×10^5 organisms.

DISCUSSION

Cortisone has a definite effect on acute infections with *Salm. typhimurium*, and modifies the mean time to death, the total mortality and the number of survivors with persistent carrier infection. When cortisone administration precedes bacterial inoculation the degree of this augmentation is greater than when it follows shortly after. This suggests that cortisone acts progressively on host factors in the

infective process rather than upon the bacteria. Preliminary *in vitro* experiments with *Salm. typhimurium* failed to show any alteration of growth rate when cortisone in concentrations comparable to those used in mice was added to the medium.

The final critical bacterial population was achieved more quickly in cortisone-treated than in untreated mice, presumably due to a decrease in the net loss per bacterial generation as a result of impaired host defence mechanisms. There was no evidence to suggest increased susceptibility to bacterial products as a cause of early death in treated animals. Although the infectivity of all three strains of *Salm. typhimurium* was increased, a relative order of virulence could still be discerned in cortisone-treated animals. Similarly, in experimental brucellosis, Spink and Anderson (1958) have shown that infection with *Brucella abortus* strain 19 is considerably enhanced by cortisone adminstration but never to the same extent as with naturally-virulent strains.

Observation of the course of intravenous infection during cortisone-treatment showed that the ability to clear organisms from the bloodstream was not impaired, nor did the animals fail to restrain bacterial growth in the organs in the first few hours. After 24 hr., however, when the organisms had presumably become adapted to their new micro-environment and had begun to multiply, the rate of increase in bacterial population was much greater in the cortisone-treated group, and the final critical bacterial population was reached more quickly than in untreated animals.

The finding of virtually-normal initial blood-clearance after intravenous infection is similar to the results obtained by Magee and Palmer (1953) and Gell and Hinde (1953) when inert particles were injected into animals treated with cortisone. The subsequent inability to deal with the organisms after clearance or to prevent bacterial re-invasion is consistent with the view of Halpern and his colleagues (Halpern *et al.* 1953 ; Biozzi *et al.*, 1953 ; Benacerraf *et al.*, 1954, that cortisone does not impair the phagocytic activity of existing reticulo-endothelial tissue but prevents the mobilization and proliferation of further cells which normally takes place after an initial uptake.

Cortisone administration to the chronic carrier had much less effect. In most animals, there was no recrudescence of infection either during treatment or as a " rebound " effect after withdrawal, nor was final eradication of infection facilitated by the drug. Organisms isolated from chronic carriers have not lost their potential virulence by antigenic degradation or mutation and have normal biochemical properties (Hobson, 1957a). Hence, it seems likely that the carrier has acquired as a result of long-continued infection defence mechanisms quantitatively or qualitatively different from those of the recently infected animal and that these cannot be inactivated by cortisone.

Evidence on the effect of cortisone in established infections is conflicting. Hart and Rees (1950) showed that chronic stable pulmonary tuberculosis of mice could be reactivated by cortisone, whereas Abernathy and Spink (1952) found little effect in guinea pigs with chronic brucellosis. Mogabgab and Thomas (1950, 1952) found that intravenous injection of haemolytic streptococci in rabbits gave a lowgrade persistent infection readily enhanced by subsequent cortisone treatment. However, animals which had been specifically hyperimmunized showed no enhancement of infection by large challenge doses of streptococci given during cortisone treatment.

These differences probably reflect not only variations in species-susceptibility to the drug, and differences in the schedule of dosage, but the artificiality of some of the experimental model infections chosen.

SUMMARY

Cortisone enhances the infectivity of *Salm. typhimurium* for mice by permitting a final critical population of organisms to be attained more rapidly than normal from a recent small inoculum.

The low virulence of two particular strains of *Salm. typhimurium* is apparently due to their rapid suppression by host factors rather than to any intrinsic inability to obtain sufficient metabolites from mouse tissues.

There is a lag period before the full biological effect of cortisone on the host-parasite relationship is exerted.

Cortisone is much less effective in disturbing the host-parasite equilibrium in the chronic carrier state than in recently-established infection.

The course of events shortly after infection in cortisone-treated animals is described, and the findings have been discussed.

I am indebted to the Medical Research Council for the provision of a grant towards the expenses of this investigation, and to Messrs. Menley and James (A. J. White) Ltd. for generous supplies of furazolidone.

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THE EFFECT OF COMPOUND 48/80 ON THE AUTOGRAFT AND HOMOGRAFT REACTION

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Received for publication November 17, 1959

THE role of histamine in the homograft reaction may be studied (a) by administering histamine, (b) by blocking its action with antihistamine drugs, and (c) by depleting reserves of histamine with liberator substances, *e.g.* compound 48/80; were histamine an important substance in relationship to the survival of a homograft, its depletion intrinsically (Dale, 1950) in the tissues might affect the survival time and histological features of the take of a homograft. Method (b) has been widely used (Foster and Hanrahan, 1948; Marconi, 1950; Stark, 1953; and Woodruff and Boswell, 1954). This paper reports experiments in which methods (a) and (c) have been used.

In rats we have made skin autografts and homografts, thereafter studying histology of the graft and graft bed, and estimating histamine in the graft bed. These experiments have been done in untreated rats, and in rats treated by compound 48/80, by histamine administration, and by an antihistaminic agent.

MATERIALS AND METHODS

Albino Wistar rats and hooded rats of a home bred strain were employed and at least 3 rats were included in each experimental group. Autografts and homografts were studied. The experimental groups were paired so that each rat acted as donor and recipient for its fellow.

Under intraperitoneal thiopentone anaesthesia the skin over the back of the neck and interscapular area was shaved, cleansed with spirit, dried, and a full thickness circular graft of skin 2·0 cm. in diameter was removed. Care was taken not to include in the graft underlying muscle fibres of the panniculus carnosus. Having been transferred to the graft bed which was also 2·0 cm. in diameter, the graft was held in place by 8–12 sterile black silk sutures placed regularly round the circumference. Many grafts were protected by plastic buttons of larger diameter than the graft, and these were held in place by 4 sutures which were placed outwith the grafted site; other grafts and adjacent skin were sprayed with "Nobecutane" (Evans). The grafts were inspected daily. Once necrosis of the graft appeared the animal was killed. A block of tissue containing graft, graft bed and adjacent healthy skin was removed, aligned on filter paper and fixed in 25 per cent picroformol for histological study. A portion of graft bed, weighing 800–950 mg. was removed for histamine assay according to the method of Feldberg and Talesnik (1953). Occasionally a portion of the graft itself was taken for histamine assay.

The release agent, compound 48/80, was given intraperitoneally or subcutaneously, the dose being 100 μg. daily × 100 μg. to a maximum of 500 μg. daily depending on the animal's tolerance. In some groups this regime stopped on the day of operation; in other groups it continued post-operatively until the animal was killed. The anti-histamine agent "Neo-

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Antergan" (Mepyramine maleate) was given intraperitoneally pre- and post-operatively the dose being 1 mg./kg./day, increasing by 1 mg./kg. to a maximum of 4 mg./kg./day. Histamine acid phosphate was given intraperitoneally, the dose given being 0·01 mg./100 g./day.

RESULTS

Autografts

Normal Animals

Histamine levels.—Since most autografts survive in normal animals, there was no indication of the correct time at which samples should be taken for histamine assay; random samples suggested that the act of removing a graft causes a temporary mild lowering of the skin histamine to the lower limit of normal in the animal and that this value gradually rises to the upper limit of normal during the healing phase. This result is similar to that noted during our experiments with wound healing (Boyd and Smith, 1959).

Histology.—On histological examination of the granulation tissue of the graft bed the most notable change was in the population of mast cells. None was seen during the first 3 days. From the 3rd to the 7th days cells with the morphological characters of mast cells but without polychromasia were seen. These cells are oval discs with an eosinophilic cytoplasm which is often granular, and a nucleus which is oval or spherical, situated near the centre of the cell. These cells could not be detected easily after the 7th day because the granulation tissue was becoming so fibrous, but on the 12th day weakly polychromatic cells appeared.

Meanwhile, in the autograft itself, mast cells were present in normal numbers at all stages except the 3rd and 4th days when they appeared to be reduced in numbers.

Infiltration of the graft bed by other cells was variable, but never massive. Polymorphs predominated and were present in moderate numbers, and eosinophils became evident in small numbers about the 6th day. It was about this time that they were recorded first in the autograft also. Round cells, including lymphocytes and plasma cells were present in very small numbers, and did not predominate over the numbers of polymorphs.

Histamine Depleted Animals

In addition to the study of autografts in normal rats, we studied the healing response of autografts in rats which had been depleted of their skin histamine by compound 48/80 given either locally or intraperitoneally. The impression gained in these 2 series was that the grafts failed to take either as rapidly or as firmly as in untreated animals. This was supported by the retarded healing and organization noted on histology. In these series, polychromatic mast cells were absent from the granulation tissue of the graft bed, but where this granulation tissue was oedematous and vascular rather than fibrous, non-polychromatic mast cells could be identified in many cases.

To summarize these findings, it would appear therefore that the results of these autograft experiments support 2 of our findings concerning wound healing (Boyd and Smith, 1959), namely that depletion of the skin histamine retards autograft healing and discharge of the polychromatic granules by the mast cell need not be followed by death of the mast cell.

Homografts

The Table summarises our experiments; at least 3 rats were used in each subgroup. The histamine values are those found in the tissue composing the graft bed at the time when the graft was seen to be completely necrotic.

TABLE.—*Survival Time and Histamine Content of Graft Bed after Various Drug Treatments of Donor and/or Recipient Rats*

(Each value is the mean of results from at least 3 rats).

Homograft experiments	Albino rat recipients		Hooded rat recipients	
	Survival of graft (days)	Histamine level (μg./g.)*	Survival of graft (days)	Histamine level (μg./g.)*
A. *Recipient untreated*				
1. Graft : normal donor	10	14·7	9	16·5
2. Graft donor : local 48/80	19	14·2	11	16·9
3. Graft donor : peritoneal 48/80	18	13·5	20	17·5
B. *Recipient : local* 48/80, *before operation*				
1. Graft : normal donor	12	14·8	15	13·8
2. Graft donor : local 48/80	23	10·1	23	7·2
3. Graft donor : peritoneal 48/80	26	8·6	17	4·8
C. *Recipient : local* 48/80, *before and after operation*				
1. Graft : normal donor	13	5·4	12	5·1
2. Graft donor : local 48/80	18	3·1	17	2·9
3. Graft donor : peritoneal 48/80	20	2·6	19	4·0
D. *Recipient : intraperitoneal* 48/80, *before operation*				
1. Graft : normal donor	20	16·6	20	15·5
2. Graft donor : local 48/80	15	14·6	28	14·1
3. Graft donor : peritoneal 48/80	18	20·2	18	10·8
E. *Recipient : intraperitoneal* 48/80, *before and after operation*				
1. Graft : normal donor	19	4·1	19	5·2
2. Graft donor : local 48/80	23	6·8	24	3·4
3. Graft donor : peritoneal 48/80	21	3·5	21	2·8
F. *Recipient : intraperitoneal histamine, before and after operation*				
1. Graft donor : peritoneal histamine acid phosphate	18	19·7	14	23·9
G. *Recipient : intraperitoneal mepyramine maleate, before and after operation*				
1. Graft : normal donor	20	21·6	22	18·2

* Histamine level in graft bed at the time of graft necrosis.

Normal recipients (Group A)

Histamine levels.—Our previous observations (Boyd and Smith, 1959) show that any reduction in histamine present at the operation area is usually slight, and is limited to the 5th to 10th days. The low values in this experiment, and especially between the ninth and twentieth days suggests that either there may be a mild diffusion of histamine from the graft bed into inactive homograft tissue which must be existing on a very reduced metabolic rate before it dies (Medawar,

1946), or into the serous exudate following the operation, or that the operation has provoked the activation of enzymes of the histaminase group in the grafted area.

General.—The survival time of first set untreated skin homografts on normal rat recipients is comparable with that reported for rats by Billingham and Parkes (1955) and with those in other species (row 1). It would appear that if the homograft is taken from an animal whose skin is depleted of histamine, the survival time is prolonged in 3 instances (rows 2 and 3).

Histology.—The histology of the skin homograft reaction is well known (Billingham and Parkes, 1955; Gibson and Medawar, 1943; Medawar, 1944) and our studies were made with the particular aim of following the mast cell and eosinophil.

Polychromatic mast cells were present in normal numbers in the skin adjacent to the graft, but were usually absent from the graft bed, and in the few cases when present they were poorly staining or non-polychromatic. If the graft itself were completely necrotic by histological standards, it was unusual to be able to make any worthwhile study, but nevertheless these occasionally revealed aggregates of polychromatic granules arranged in such a fashion that it was possible to suspect their origin from mast cells. In other less necrotic grafts polychromatic mast cells were identified with greater certainty—even when the graft donor had received a pre-operative course of compound 48/80—and they were present in reduced numbers in these circumstances. This would suggest that the mast cells of the homograft had become partly replenished in polychromatic material during the interval between operation and graft necrosis. This finding coincides with the low histamine values obtained from the graft bed, and would support one of the possible explanations given above for this.

Compared with the autograft response, there was as a rule a much greater cellular infiltration in the bed of the homograft. Polymorphs and eosinophils appeared to be just as numerous as lymphocytes and plasma cells. Study of the eosinophil population in these homografts suggests that it was unaffected by the procedures involved.

Histamine depleted recipients (Groups B–E)

Histamine levels.—Generally the histamine levels vary according to the amount of "treatment" given to the animals. When the course of compound 48/80 was stopped on the day of operation (groups B and D) the histamine levels rose post-operatively towards normal limits but remained lower than the levels we had found earlier in wounds and in the autograft series. Possible explanations for this have been suggested above.

General.—Homografts from normal rats, when applied to rats in whom compound 48/80 has been given directly into the recipient area, survive slightly longer than those applied to rats without these preliminary injections. Similar grafts applied to rats submitted to intraperitoneal compound 48/80 survive much longer.

The use of homografts from histamine depleted donor rats increases further the survival time significantly in the case of animals which have been depleted locally only (groups B2 and 3, C2 and 3), and to a less significant extent in the case of rats whose skin has been depleted of histamine by intraperitoneal injections

of compound 48/80 (groups D2 and 3, E2 and 3). The action of compound 48/80 appears to be exerted pre-operatively because the results suggest that continuation of the injections post-operatively, irrespective of the route, makes no difference to the survival time of the homograft (groups B and D compared with groups C and E). This result also supports our findings with regard to wound healing.

Histology.—Where the course of compound 48/80 had been stopped on the day of operation, the polychromatic mast cell content of the adjacent undamaged recipient skin had returned to normal by the time the applied homograft had become necrotic. Where the drug was continued post-operatively, polychromatic mast cells were absent from the unaffected skin, or were present in very reduced numbers.

As in the cases of autografts and normal (untreated) homografted rats, polychromatic mast cells were absent as a rule from the granulation tissue of the graft bed, although in one or two individual cases an occasional weakly polychromatic mast cell could be found. However, when the granulation tissue was vascular and oedematous, non-polychromatic mast cells could be identified in H. and E. sections, but this became impossible as the graft bed became more fibrous.

If the graft was not entirely necrotic, polychromatic mast cells, or aggregates of polychromatic granules, could be found not only in those grafts taken from normal (untreated) donors, but also in several grafts from donors which had been depleted of their skin histamine pre-operatively. This was particularly so in groups B and D, where the recipient's course of compound 48/80 was stopped at the time of operation, but was also noted in an occasional instance in groups C and E where the recipient's course of compound 48/80 continued post-operatively up to the day of death. In other cases however, the graft was completely necrotic and no useful observations could be made.

The exudation of cells in the granulation tissue seemed to consist equally of polymorphs and eosinophils on the one hand, and lymphocytes and plasma cells on the other. The numbers of eosinophils did not appear to be affected by the type of course of compound 48/80 given to the recipient or by the type of homograft applied.

Summary of these homograft experiments suggests that utilising a graft from a donor rat depleted of its skin histamine by compound 48/80 leads to a prolonged survival time; that depleting a recipient rat of its skin histamine by compound 48/80 leads to a prolonged graft survival time; that depleting both donor and recipient rat has the same effect as either alone and does not produce an additive or synergistic effect; and that compound 48/80 appears to cause its effects pre-operatively, and it is not necessary to prolong the course into the post-operative period.

Control Homograft Experiments with Injections of Histamine or an Antihistaminic Agent

Recipients given histamine acid phosphate intraperitoneally before and after operation (Group F).

Histamine.—The histamine level of albino rat recipients is at the lower limit of normal. The dose employed was 0·01 mg./100 g./day. The mean value

obtained in hooded rat recipients is at the upper limit of normal for the back of the neck.

General.—The survival times are less than have been found in the bulk of homograft experiments already cited above, although they are nevertheless longer than the natural survival time of 9–10 days.

Histology.—As already observed in the wounds series (Boyd and Smith, 1959) an intraperitoneal course of histamine causes enlargement of the mast cells in the dermis of the adjacent normal skin with an increase in the number and size of their granules which appear to give a deeper polychromatic reaction than normal. It was also observed that weakly polychromatic mast cells could be identified more constantly in the granulation tissue of the graft bed although they were present in smaller numbers compared with the adjacent skin but in greater numbers than observed in the graft beds of the other experiments reported above. Since the graft donors had been given a course of histamine acid phosphate intraperitoneally, it was to be expected that, in suitable cases, polychromatic mast cells were identifiable in partly necrotic grafts.

The cellular response in the graft bed was equally composed of polymorphs on the one hand and lymphocytes and plasma cells on the other. Eosinophils were absent or present in very reduced numbers in this series.

Recipients given "Neo-Antergan" (mepyramine maleate) intraperitoneally before and after operation (Group G)

Histamine levels.—The action of mepyramine maleate must be different from that of compound 48/80, for in spite of a continuous pre- and post-operative intraperitoneal course of the drug the histamine levels within the graft bed are within normal limits.

General.—This treatment compares favourably with that of group E above, and permits the survival time of a homograft from a normal (untreated) donor rat to be doubled. This is contrary to Marconi's results with guinea-pig homografts (Marconi, 1950).

Histology.—The normal skin adjacent to the graft bed shows a reduction in the number of polychromatic mast cells present. This reduction is about midway between the numbers normally observed and the reduction effected by compound 48/80. Further it appears to select the mast cells in the upper half of the dermis (*i.e.* in the dermal papillae mainly) while those in the lower half at the level of the hair roots appear to be spared. This may be the explanation of the normal histamine values obtained, for the graft bed involved the lower half of the dermis. At the same time, since homografts from normal (untreated) donors were employed some of the graft histamine may have diffused into the graft bed. If this is possible, it is unlikely that histamine from the graft contributes greatly to the actual levels observed, being merely a gradual mild diffusion from a small region of normal concentration to a larger region of reduced concentration.

In other respects the histology of these grafts is similar to those of homografts discussed above.

Summary of these 2 groups shows that the histological observations in the histamine treated group are consistent with the results obtained previously from wound studies; that mepyramine maleate intraperitoneally produces the same clinical effect in the rat as compound 48/80; and that the action of mepyramine maleate appears to be different from that of compound 48/80.

DISCUSSION

These experiments yield several points for discussion. It is apparent that lowering the skin histamine by a course of compound 48/80 lengthens the survival time of homografts to about twice the normal length, but does not allow the animal to accept the graft permanently. It can be deduced from this that histamine is not an essential factor in deciding the fate of a homograft. The graft is rejected later and compound 48/80 appears to be effective only in the early days of the transplant before resynthesis of histamine has taken place. Another important factor may be attributable to the delayed granulation tissue response noted in a series of experimental wounds (Boyd and Smith, 1959) in animals injected with compound 48/80 ; the prolongation of wound healing may delay any immunological response by the recipient for it may result in a lag in the development of lymphatics through which antigenic material from the graft may be transmitted to the regional lymph nodes, and in vascular channels through which antigens may reach the systemic reticulo-endothelial system (Medawar, 1944; Scothorne and McGregor, 1955). It may equally delay any sensitivity response which the homograft may initiate.

Another aspect of the action of compound 48/80 is also concerned with the degranulation of the mast cells. These granules, beside containing histamine, also contain heparin, and other substances which may play some part locally in retarding the antigen-antibody response. Compound 48/80, while being a histamine releasing agent, also involves other constituents of the mast cell.

It has been known that cortisone may delay wound healing (Ragan, Howes, Plotz, Meyer and Blunt, 1949) and it has also been shown that this drug prolongs first set homograft survival whether it is administered systemically or locally (Billingham, Krohn and Medawar, 1951*a* and *b*). It is tempting to suggest that this action of cortisone, like that of compound 48/80, is effected through the mast cell and skin histamine, or vice versa. The knowledge that locally applied cortisone fails to act in the case of second set homografts (Billingham *et al.*, 1951*b*) suggests that this may indeed be the main action of cortisone in first-set homograft reactions. With second-set grafts the antigen-antibody reaction may dominate. A.C.T.H. administration also prolongs homograft survival, as does pregnancy (Medawar and Sparrow, 1956), and it is probable that each of these acts through the same channels as cortisone.

The end result of mepyramine maleate ("Neo-antergan") treatment appears to be similar to that of compound 48/80 in prolonging the homograft survival time in rats, but our histamine values in the graft bed at the time of graft necrosis, and the histology, both suggest that the mode of action of this drug is different from that of compound 48/80. While compound 48/80 acts on all mast cells of the dermis, mepyramine maleate appears to select only those mast cells in the superficial half of the dermis. Both drugs cause the affected mast cells to lose their polychromatic material. This finding may be a clue to the varied effects of different antihistamine drugs on the homograft reaction, for instance the apparent benefit derived from tripellenamine ("Pyribenzamine") in a human case of skin homotransplantation (Foster and Hanrahan, 1948) and the absence of any benefit from promethazine hydrochloride ("Phenergan") on skin and thyroid homografts in the guinea-pig (Woodruff and Boswell, 1954). Further support is given to this suggestion by the observation that the guinea-pig is tolerant of promethazine

hydrochloride, but is quite intolerant of compound 48/80 (Smith, unpublished). Indeed, Marconi (1950) reported that " Neo-antergan " is ineffective in prolonging the survival of skin homografts in the guinea-pig. It would seem from these results that the guinea-pig is an unsuitable animal for testing the effectiveness of antihistamines on skin homografts. The explanation of this may be that its skin histamine values are too low (Feldberg, 1953).

Lastly, Mota (1959) has recently studied the effect of antigen and octylamine on mast cells in sensitized guinea-pigs. He suggests that most of the histamine released in an antigen-antibody reaction comes from the mast cells, and it seems likely that these cells are concerned with the uptake of antibodies.

Do the above results help further in elucidating the nature of the homograft reaction? The argument used originally by those who introduced antihistamines into the study of the homograft response was that if sensitivity played any part in this, then this therapy would counter any released tissue histamine as a result of the antigen-antibody combination. It would appear however that our experiments show that compound 48/80 exerts its main effect in the early period of the experiment, even pre-operatively, long before any antibody could have been formed by the host against first-set graft antigen : the same argument applies to mepyramine maleate. Therefore it would seem that another conclusion, drawn from our wound experiments, is applicable to the present homograft experiments, namely that histamine is essential in the early phases of the granulation response of the graft bed of the recipient to the graft. Our experiments would appear therefore to have no direct bearing on any immunological-hypersensitivity reaction elicited by these homografts. At the same time however attention must be given to the fact that promethazine hydrochloride (" Phenergan ") has been used effectively under experimental conditions to control the Arthus reaction, and the Shwartzman phenomenon (Benacerraf and Fischel, 1949) and egg-white hypersensitivity in rats (Feldberg, 1953). Not every antihistamine drug possesses these properties ; for example, tripellenamine (" Pyribenzamine ") has no effect on the Arthus reaction (Fischel, 1947). The whole field therefore is very confusing, not least being the definition of what constitutes an antihistamine. There is also controversy over the fundamental nature of the homograft reaction itself. Evidence has been accumulating in recent years, mainly from Medawar's department, that the homograft reaction is essentially an antigen-antibody reaction of predominantly local origin and of cellular nature in support of Loeb's beliefs (Loeb, 1930, 1937), although systemic effects cannot be neglected. It is considered that the homograft reaction may be compared with the Arthus phenomenon (Rogers, 1950; Woodruff and Boswell, 1954). While we set out with these facts in view, our experiments suggest that compound 48/80 and mepyramine maleate act in the rat at a period before any sensitivity reaction could be effective.

The expected action of histamine in hastening homograft rejection, as it hastened wound healing, did not materialize. In our experiments eosinophils were neither numerous nor absent in the autograft series or in the series of untreated (normal) rats receiving normal homografts. The series which showed a reduced eosinophil population in the graft bed was the one treated pre- and post-operatively with intraperitoneal injections of histamine acid phosphate. Here, the findings were not as clear cut as they were in our wound series (Boyd and Smith, 1959) and we suspect that this may be due to complicating factors, known and unknown, in the present series which were absent in the wound

series. The population of eosinophils in the graft bed was not exceptional in the other experiments of this series.

SUMMARY

A main conclusion from these experiments is that histamine plays no essential part in the homograft reaction, but that its depletion in the skin by retarding the normal granulation tissue response for healing may postpone thereby the circumstances which are normally necessary for the homograft reaction.

The rate of healing of autografts in rats is retarded when the skin reserves of histamine are depleted by a histamine release agent, compound 48/80. On the other hand, this treatment increases the survival time of homografts.

It appears to be more important to administer the drug (compound 48/80) pre-operatively than post-operatively, and it appears to be as effective when applied locally as it is when given intraperitoneally.

The homograft survival time can be somewhat extended by depleting the donor the recipient rat.

Mepyramine maleate (" Neo-antergan ") given to the rat causes the same effect but appears to act differently from compound 48/80 for the latter degranulates all mast cells of the dermis, whereas the former only acts upon those mast cells in the upper half of the dermis.

Discharge of the polychromatic material by the mast cell is not necessarily followed by its death.

The action of Cortisone in prolonging the skin homograft survival time may be mediated through the mast cell and the skin histamine rather than by preventing antibody formation primarily.

The eosinophil does not appear to play a major part in the homograft reaction.

The administration of histamine pre- and post-operatively causes enlargement of the mast cells of the skin with increased numbers of polychromatic granules. Histamine does not shorten the homograft survival time especially when the graft donor has been treated similarly. This is not in full agreement with our experience of wound healing.

We are very grateful to Professors C. F. W. Illingworth and D. F. Cappell for their encouragement during the performance of this work, and to Dr. A. C. White of the Wellcome Research Laboratories for a supply of compound 48/80. We are grateful to the Department of Health for Scotland for a grant to one of us (A. N. S.).

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STUDIES ON TRANSFERENCE OF BONE

I. A Comparison of Autologous and Homologous Bone Implants with Reference to Osteocyte Survival, Osteogenesis and Host Reaction

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Received for publication November 23, 1959

Despite the large volume of clinical and experimental work related to the fate of bone grafts, very little is known about either the extent of their survival or the manner in which the graft becomes incorporated into the host skeleton (Dempster, 1958; Chase and Herndon, 1955). It is widely accepted, however, that both in man (Longmire, Cannon and Weber, 1954; Phemister, 1951) and experimental animals (Enneking, 1957) autografts are more successful than homografts, *i.e.* they are incorporated earlier and more completely into the host skeleton.

Since homologous bone is less readily acceptable than autologous bone, one is tempted—on analogy with other tissues (Medawar, 1958)—to attribute this to the cellular component rather than to the matrix. Observations on the extent of cell survival in bone grafts are however vague, and often merely speculative. If it is true that these grafts act only as a scaffolding on which new bone is laid down (Hyatt, 1954)—*i.e.* their component cells do not actively influence the behaviour of the graft—then the readier acceptance of autologous bone must be attributed to some specific feature of the matrix—a relatively unorthodox concept in the field of tissue transplantation. Opposing the purely static function of bone grafts, however, Phemister (1951) ascribes the superiority of autografts to the better survival of cells within them. Likewise Ham and Harris (1956) maintain that the osteocytes of autografts may survive for a time, whereas homologous osteocytes are destroyed. While it is generally believed that transplanted autologous cells will survive longer than homologous cells, it is doubtful whether this contention is entirely applicable to bone. The work of Chalmers (1959) and also our own findings (Heslop, Zeiss and Nisbet, 1959) show that osteocytes occupy a protected position inaccessible to the host inflammatory cells, and being virtually within a diffusion chamber are unlikely to succumb to the homograft reaction (Algire, Weaver and Prehn, 1957).

The ready formation of new bone round autografts is a well recognised phenomenon, and is essential if the graft is to " take ", but the reason for the superiority of autografts over homografts in this respect remains uncertain. Where autologous grafts contain potentially osteogenic cells, such as periosteum, endosteum or marrow, it is not unreasonable to explain osteogenesis as resulting from survival and differentiation of these cells. In the case of osteogenesis associated with autografts containing cortical bone only (De Bruyn and Kabisch, 1955), the mechanism remains obscure. The manner in which bone formation is impaired

around homografts likewise requires elucidation. It is tempting to suggest that the homograft reaction—in some way hitherto unexplained—interferes with osteogenesis round homografts. However, the nature of the homograft reaction around bone has received scant attention.

Unfortunately it is difficult to compare the various results available, since experimental grafts have been of widely differing sizes, and even more important, of widely differing tissue constitution. As Weinmann and Sicher (1955) point out, " bone is a tissue ; bones are organs ". Accordingly results obtained from grafting pure bone (as far as this is possible) are unlikely to be strictly comparable with those obtained from grafts which contain an assortment of potentially osteogenic soft tissues.

In consideration of these difficulties the present experiment was carried out in order to elucidate the following points :—(1) the extent to which autologous and homologous osteocytes survive, (2) the extent to which osteogenesis can be correlated with osteocyte survival, and (3) the nature of the homograft reaction round bone.

In order to obtain comparable results standard tubular portions of cortical bone were used. Soft tissues were removed as thoroughly as possible without interfering with the viability of the osteocytes. A standard extraskeletal site was chosen in order to avoid confusion arising from osteogenesis associated with skeletal trauma.

MATERIALS AND METHODS

Animals.—Young adult rats were used throughout the experiment. Wistar rats were used for the autologous series. In the homologous series Wistar rats received grafts from hooded donors. That the two strains were unrelated genetically was shown by the rejection of 1 × 1 cm. skin grafts from hooded to Wistar rats in 9·4 days. Rejection time was calculated using the method of Taylor and Lehrfeld (1953).

Preparation of implants.—These were prepared with dental excavators and reamers, and consisted of tubular pieces of tibia 0·5 to 1·5 cm. in length. Measurements were made from the proximal tibial epiphysis. The periosteum and medullary tissues were thoroughly scraped from all implants.

Site of implantation.—The implants were inserted between the dermis and panniculus carnosus muscle. Each Wistar rat received two separate tubular implants. A given animal received either homografts or autografts—never both.

Time of excision.—In both autologous and homologous series the implants were excised for histological examination 2, 3, 4, 7, 10, 12, 14, 35, 63, 100 and 250 days after insertion.

Number of specimens.—On each of the above days 4 to 10 specimens were examined histologically. A total of 141 specimens was examined, several sections being available from each implant.

Extent of excision.—In all cases the whole implant was excised together with the surrounding soft tissues.

Preparation of specimens for histological examination.—Material was fixed in 10 per cent neutral formalin, decalcified in 7·5 per cent nitric acid and embedded in paraffin. Sections were cut at 5 μ and stained routinely with haematoxylin and eosin. Selected sections were stained with Van Gieson, toluidine blue, pyronin-methyl green, Lendrum's method for eosinophils (Clayden, 1955), P.A.S. and Congo red.

Counting of osteocytes.—The relative number of osteocytes surviving in the implant was estimated by counting six standard high power fields in the manner shown in Fig. 1. The number of lacunae containing morphologically normal osteocytes was expressed as a percentage of the total number of lacunae. Osteocytes showing appreciable chromatolysis or pyknosis were regarded as non-living. The number of lacunae available for counting by this method varied between 200 and 350. Counting was restricted to the lacunae of the original implant and excluded those of any new bone formed after implantation. The percentage of osteocytes in similarly prepared fresh cortical bone was also estimated in this way.

Estimation of rate of autolysis of osteocytes.—Twenty-one tubular portions of tibia were prepared in the same way as the implants. These were placed in Petri dishes in a 37° oven and kept moist with saline. Three specimens were removed from the oven and fixed in

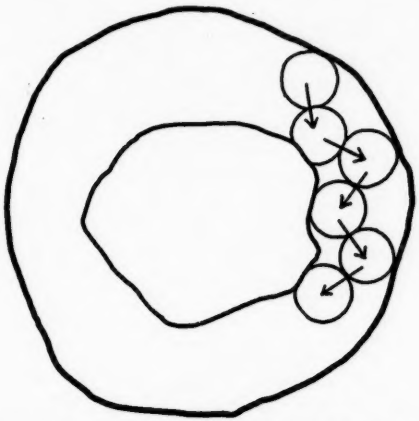


FIG. 1.—Method of assessing the relative extent of osteocyte survival in the implants. All the lacunae were counted in six consecutive high power fields, in the order illustrated. The number of normal osteocytes was expressed as a percentage of the number of lacunae.

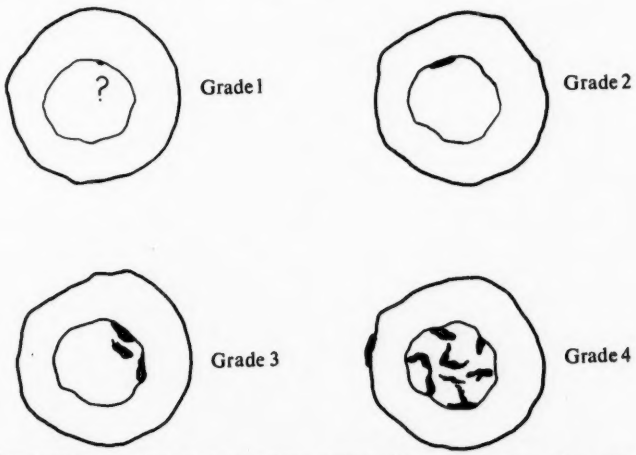


FIG. 2.—Quantitative grading of osteogenesis during the first 35 days after implantation. The quantity of new bone outside the implants relative to that within the medullary cavities showed approximately the proportion illustrated in Grade 4.

formalin at times corresponding to the excision of implants in the main experiment, *i.e.* 2, 3, 4, 7, 10, 12, and 14 days after preparation. Specimens were prepared for histology in the same way as the implants and subjected to the same counting technique.

Quantitative estimation of new bone formation.—An arbitrary grading system was evolved (Fig. 2) based on the total quantity of new bone visible in the vicinity of the implant. Its

use was restricted to the first 35 days after implantation, as subsequent remodelling of the new bone brought about a quantitative diminution.

Injected specimens.—Some of the animals from this experiment were killed and the tissues injected with Berlin blue, in order to study the vascularization of the implants. The techniques used in this connection are described elsewhere (Zeiss, Nisbet and Heslop, 1960).

RESULTS

Survival of osteocytes

Sections taken from fresh bone prior to implantation showed an average of 94 per cent of the lacunae to contain morphologically normal osteocytes in sections cut at 5 μ. The cells were evenly distributed throughout the specimen in sections of uniform thickness. In a few sections where the thickness was uneven the proportion of empty lacunae increased at the tapering extremities. Similarly in very thin sections there was a higher percentage of empty lacunae.

The distribution of osteocytes in the implants remained for the most part fairly uniform during the first 14 days in both series. In a few specimens where the distribution of living cells was patchy, they showed no regular predilection for any particular part of the implant and could not be correlated with the apparent vascularization of the specimen. Counting of osteocytes by the method illustrated in Fig. 1 showed a progressive diminution over this period in the number of lacunae containing morphologically normal cells. The average percentages of surviving cells remained closely similar during the first week in both series. After the end of the first week, however, the autologous series came to show a slightly higher average proportion of living cells than did the homologous series (Fig. 3).

EXPLANATION OF PLATES

FIG. 4.—Homograft 63 days. Osteocytes are seen surviving round the outer circumference of the implant, where there are patchy aggregations of lymphocytes. The connective tissue in the medullary cavity has a characteristic lacy appearance. ×48.

FIG. 5.—Homograft 250 days. Osteocytes surviving near the outer circumference of the implant. ×360.

FIG. 6.—Autograft 14 days. There is extensive osteogenesis within the medullary cavity, with associated development of haemopoietic marrow. ×60.

FIG. 7.—Autograft 250 days. Widening of bone canals, many of which show circumferential deposition of new bone. Layers of new bone have also been deposited on the surfaces of the implant. ×53.

FIG. 8.—Homograft 250 days. The quantity of new bone associated with this specimen represented the maximum seen in the whole homologous series. Beading of many of the collagen fibres is pronounced and in some areas gives a false impression of cellularity. (Vessels injected with Berlin blue.) ×54.

FIG. 9.—Homograft 100 days. Amorphous calcified material within medullary cavity. ×150.

FIG. 10.—Autograft 35 days. Area of connective tissue in medullary cavity which has not undergone ossification. Collagen fibres all tend to run in a similar direction. (Vessels injected with Berlin blue.) ×45.

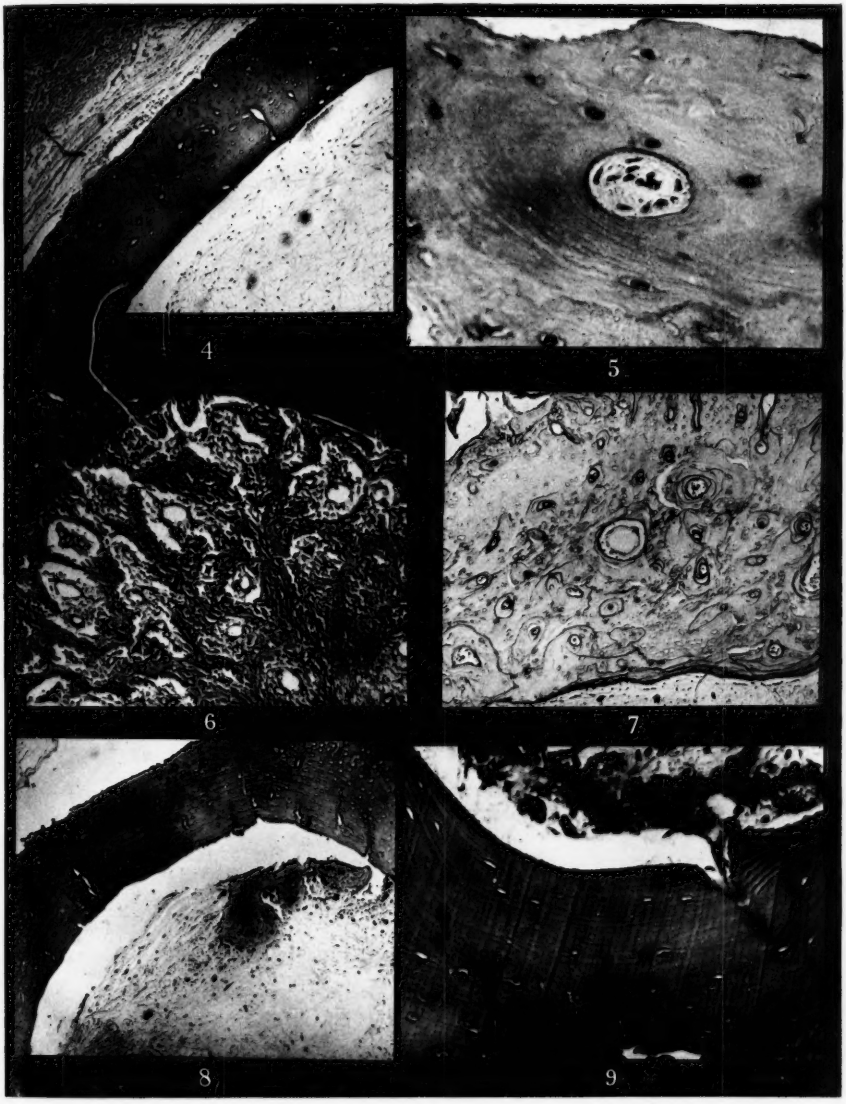
FIG. 11.—Homograft 12 days. Connective tissue in medullary cavity of implant infiltrated with lymphocytes. (Vessels injected with Berlin blue.) ×50.

FIG. 12.—Homograft 100 days. Mast cells (arrows) in loose connective tissue. Occasional fibroblasts tend to be a little plump and darkly staining. ×280.

FIG. 13.—Homograft 35 days. Collagen fibres tend to be widely spaced and run in all directions. (Vessels injected with Berlin blue.) ×180.

FIG. 14.—Homograft 250 days. Collagen fibres show characteristic beading and are widely separated. ×336.

FIG. 15.—Homograft 100 days. Collagen fibres show open lacy appearance. Blood vessels show hyaline thickening of their walls. (Vessels injected with Berlin blue.) ×140.



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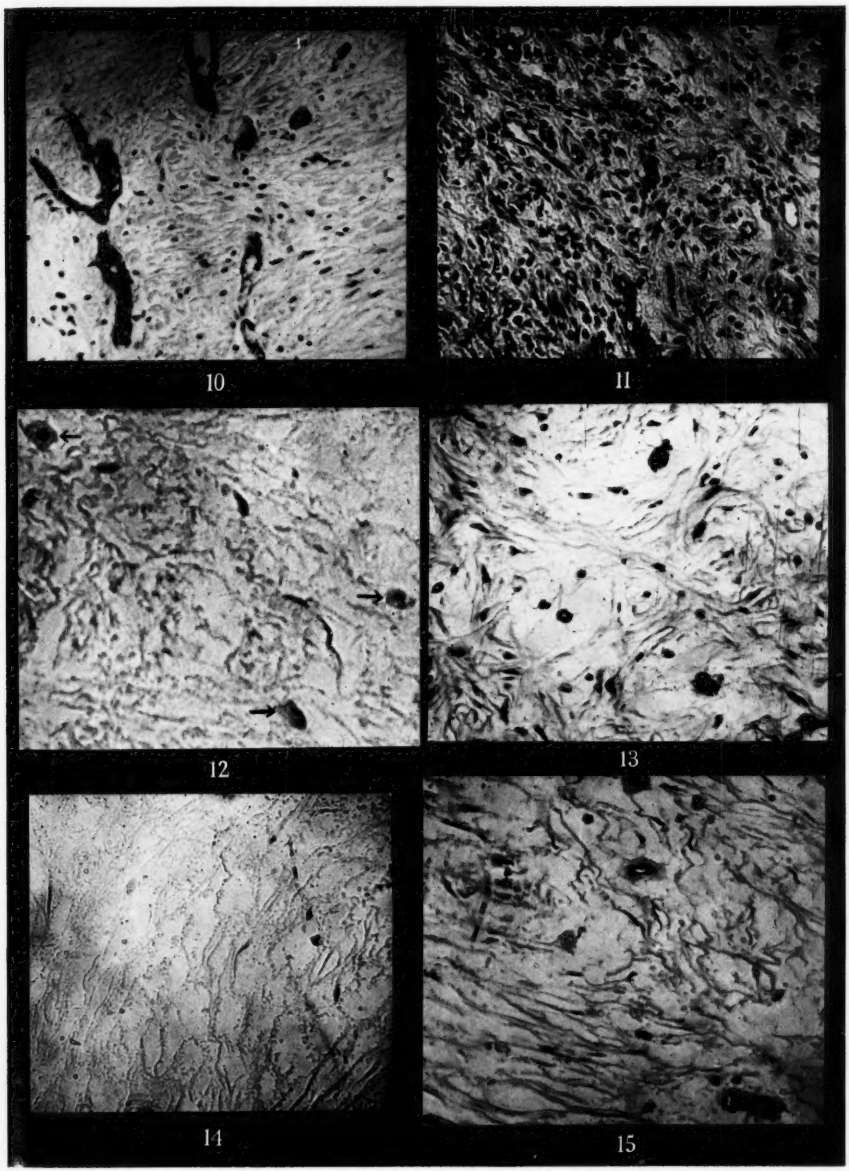

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By 35 days in both series, the surviving osteocytes invariably showed a predilection for the outer part of the implant, where they were seen as a band round the outer circumference of the bone. The width of this band of surviving cells varied from specimen to specimen and even in different parts of the same implant, but was never seen to exceed approximately 300 μ. Usually it was much narrower than this. In spite of the similarity in distribution of surviving osteocytes in both autologous and homologous implants, the average proportion of living cells remained considerably higher in the autologous group, the difference between the two means at 35 days being significant ($P < 0{\cdot}01$).

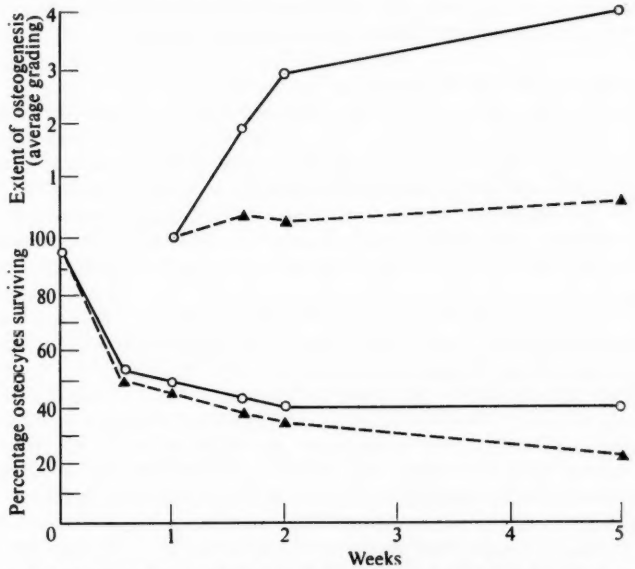

FIG. 3.—Survival of osteocytes correlated with the average extent of osteogenesis in autografts and homografts during the first 5 weeks after implantation.

○——○ Autologous
▲——▲ Homologous

These differences were maintained at 63 days, and the peripheral distribution of cells was even more in evidence (Fig. 4).

At 100 and 250 days counting of osteocytes was no longer feasible in the autologous implants. The new bone associated with the latter had matured to an extent which made it difficult to distinguish with any certainty between the cells of the original implant and those of the new bone. This problem presented little difficulty earlier in the experiment, since differences in maturity between the implant and the new bone were sufficient for identification of the former.

In homologous implants, however, the new bone seen at 100 and 250 days was still inconspicuous if present at all, and identification of the osteocytes of the original implant was relatively straightforward (Fig. 5). The average percentage

of osteocytes surviving at 100 and 250 days was estimated by the method described to be 16 and 17 per cent respectively in the homologous series.

From the 10th day onwards it was apparent in routine histological preparations that over the whole series the autografts were better vascularized than the homografts. This was subsequently confirmed by injection studies (Zeiss *et al*, 1960). In individual cases, however, particularly in the earlier specimens, it was seldom possible to correlate osteocyte survival with the apparent blood supply.

The rate of autolysis of osteocytes in comparable portions of bone moistened with saline and kept at body temperature, differed strikingly from the rate of disappearance of osteocytes in the implants. Chromatolysis was widespread on the second day and nuclear dissolution was complete 48 hr later. The lacunae on the fourth and subsequent days were invariably empty.

Osteogenesis associated with implants

Autologous series.—In the autologous series new bone was first seen on the seventh day, and was identifiable in the majority of specimens by the end of the first fortnight. Osteogenesis at this stage occurred in apparently circumscribed islands, which were initially always surrounded by loose vascular fibrous connective tissue. New bone formation was never seen in the absence of granulation tissue in the earlier specimens, although the later formation of haemopoietic and fatty marrow brought about an apparent reduction in the quantity of this tissue. The osteogenic tissue showed a distinct predilection for the medullary cavity of the implant—a region which had initially been hollow. The occasional osteogenic islands associated with the outer circumference of the implant were relatively infrequent by comparison.

In those specimens where new bone was seen on the outer aspect of the implant, it was usually found to occur in one of the following situations :—(1) Occupying the larger concavities in the external contours of the implant. While most of the surface of the rat tibia is convex, the lateral surface is concave. New bone was occasionally seen in loose connective tissue occupying this concavity. (2) Related to the orifices of the larger bone canals. The proximal end of the rat tibia contains substantially larger canals than does the distal extremity. It was not uncommon to find new bone being formed in the connective tissue occupying the larger canals. Osteogenesis was never seen in the smaller canals, however, till the later stages, when remodelling had occurred. (The effect of such remodelling was to convert a small canal into a larger one). (3) Near the lateral poles of the implant. The implant was round or oval in transverse section. It was inserted along a horizontal track, at the lateral margins of which there was a potential or actual space created by separation of the host tissues. The connective tissue in this situation occasionally contained small quantities of new bone.

By the end of the first fortnight the majority of the autologous implants showed abundant young fibrous tissue to have invaded the bony tubes, and the average quantity of new bone present was arbitrarily graded as 2·5 (Fig. 2). Haemopoietic marrow was associated with the new bony trabeculae in the majority of specimens (Fig. 6).

By 35 days the quantity of new bone in the medullary cavity of the tubular implants had increased, the grading being 4 in each case. Cartilage was occasionally found, but over the whole series it was very much less in evidence than bone. The soft tissues in the medullary cavity now consisted largely of haemopoietic

marrow. The new bone remained relatively immature, showing the characteristic features of woven bone, and no lamellar structure. There was a complicated system of islands and trabeculae, and the volume of new bone in the medullary cavity of the implants was greater than the volume occupied by the trabeculae of the normal rat tibia. The new bone related to the outer circumference was relatively inconspicuous by comparison. It was noted at this stage that most of the new bone was related to that part of the implant where survival of the original osteocytes was at a minimum, *i.e.* the vicinity of the inner circumference. At 35 days slight widening of the Haversian and Volkmann canals of the original implant was just perceptible. Widening of these canals never took place unless ingrowth of soft tissues had occurred.

At 63 days remodelling of the new bone had commenced in the autologous series, as a result of which the absolute quantity of new bone was considerably reduced. The quantitative grading used previously was thus no longer applicable. Trabeculae were more widely spaced and showed some identifiable lamellar structure, although much woven bone still remained. The inner aspect of the original implant showed a circumferential growth of new bone, which was, however, easily distinguishable from the original implant. The tissue between the trabeculae was now predominantly fatty, although a few haemopoietic foci persisted. The Haversian and Volkmann canals of the implant had become still further widened and contained a little loose vascular fibrous tissue, but as yet negligible new bone.

By 100 days the bone in the medullary cavity of the implant showed further maturation, but the most striking difference between these and the earlier specimens lay in the extension of the osteogenic process along the now considerably widened canals of the original implant. Similarly, there was an increase in the new bone around the outer circumference of the implant.

Changes in the 250 day autologous implants consisted mainly in the growth of the bone within the widened Haversian and Volkmann canals (Fig. 7), and also round the outer circumference of the implant. By this stage the original hollow tube contained a sparse system of trabeculae with intervening marrow. The latter was usually fatty. In addition there were circumferential rings of maturing bone on the inner and outer aspects of the implant, which were connected with each other by columns of new bone surrounding the widened Volkmann and Haversian canals. It was estimated that at least one third—and possibly more—of the specimen consisted of bone formed subsequently to implantation.

Homologous series.—Throughout the homologous series bone formation was very much less in evidence than in the autologous series (Fig. 3). Even after 250 days (Fig. 8) the quantity of bone formed in relation to homografts was considerably less than that seen round the average 14 day autograft (Fig. 6).

Initially new bone was seen in the vicinity of the homografts by the 7th day, although this was sufficiently small in quantity to be classified as doubtful. Similar minute areas, which were interpreted as probably being indicative of the earliest osteogenesis, were seen in a few specimens on the 10, 12 and 14th days, but very little increase was evident by the 35th day. The size of these islands of " new bone " was such that no comment on their vitality was practicable because of the relatively few cells contained therein.

At 63 days 3 of the 6 homologous implants examined showed no new bone at all. The remaining 3 specimens contained minute quantities of new bone, which

showed no living cells within their lacunae. The outer margins of their trabeculae were likewise devoid of osteoblasts.

No increase in osteogenesis was evident at 100 days. Of the 4 homologous implants examined at 100 days, 3 contained little recognisable bone, but showed instead small aggregations of granular calcified material which was devoid of cells (Fig. 9). Similar material had been noted occasionally in the earlier homografts, but in considerably smaller quantities than were seen at 100 days.

Even at 250 days the quantity of new bone remained small (Fig. 8), and in some specimens doubtful. Where definite new bone was present it was possible to identify osteocytes in some of the lacunae. The proportion of empty lacunae was however greater than had been noted in the new bone formed in association with autografts. Living osteoblasts round the periphery of the bony deposits were likewise a relatively less prominent feature of homografts than of autografts.

During the first 5 weeks after implantation the small quantity of new bone seen in association with the homologous implants was found mainly in relation to the outer circumference of the implant. Considerably more osteogenesis was seen round the outer circumference of the autografts, although the quantity of new bone here was negligible by comparison with that present in the medullary cavity of the autologous implants. In the later homografts the new bone was found predominantly in the tissue in the medullary cavity of the implants, relatively little having been added to the outer circumference.

Throughout the whole homologous series no widening of the Haversian and Volkmann canals was evident at any stage, the size of the bone canals at 250 days not being perceptibly larger than those examined at 2 days.

Cellular reaction around implants

Autologous series.—Specimens examined on the second day after implantation showed some congestion and oedema of the surrounding tissues, and a moderate infiltration with polymorphs and large mononuclears. By the third day the relative numbers of polymorphs had diminished slightly and small numbers of lymphocytes were seen. Large mononuclears were still in evidence and a few young fibroblasts were now identifiable. It was usually difficult, however, at this stage to distinguish between the two latter cells. Mast cells were very slightly in excess of the number usually seen in rat skin in this site. By the 4th day the proportion of lymphocytes had increased and polymorphs were relatively inconspicuous. Fibroblasts were now more easily identifiable as such, and there was a corresponding diminution in the number of cells designated large mononuclear. The cellularity around the autologous implants was at its maximum during the third and fourth days after implantation.

From the 7th day onwards, a progressive diminution in cellularity was noted, the inflammatory cells being replaced by progressively more mature fibrous tissue. Apart from the fibroblasts associated with the latter, the cell population was predominantly lymphocytic, with a few large mononuclears and very occasional plasma cells. Mast cells showed an approximately normal distribution.

By 14 days inflammatory cells of all kinds had almost disappeared from the outer circumference of the implant, which was now surrounded by fibrous tissue of only moderate cellularity. The autologous specimens examined at 35 days and later after implantation were devoid of inflammatory cells round the outer circumference. The cellularity of the fibrous tissue had decreased, and except for

the circumferential direction of the collagen fibres, the original reactive tissue was now indistinguishable from the adjacent tissues.

The sequence of events described above was seen round the convex surface of the implants. Simultaneously the concavities came to be occupied by vascular granulation tissue, which was relatively devoid of inflammatory cells after the first week. The concavities occurred : (1) In the hollow centre of the tubular implant. (2) In the concavity seen on the lateral aspect of the tibia. (3) In the larger bone canals contained within the proximal extremity of the rat tibia. (4) At the lateral margins of the implants.

During the first 14 days the fibroblasts of the granulation tissue in the medullary cavity appeared relatively less mature than the fibroblasts related to the convexities of the implant, and the tissue as a whole was relatively looser and much more vascular. On account of the fact that the transverse sections examined histologically were to a large extent random, it was not possible to estimate exactly the rate of growth of the granulation tissue down the hollow tubes. By 35 days, however, numerous sections from the autologous specimens all showed complete filling of the hollow centre. In thick sections from some of these specimens examined in order to assess vascularity, complete filling of the medullary cavity was noted at 35 days in all sections (Zeiss *et al.* 1960). The maturation of the fibrous tissue within the concavities was not strictly comparable with that of the tissue surrounding the outer convex surface of the implant. Osteogenesis was usually seen at an early stage within the granulation tissue in the concavities, and new bone formation was commonly accompanied by the development of haemopoietic or fatty marrow. Consequently the fibroblastic tissue in these sites was largely replaced while it was still relatively immature. However, the occasional small quantity of fibroblastic tissue which did not undergo early metaplasia (Fig. 10) showed no morphological abnormality at any stage. Similarly, the new blood vessels related to the autogenous implants were morphologically normal.

Giant cells which resembled osteoclasts were not seen earlier than the 4th day after implantation, and were found only with difficulty before the 10th day. From the 10th day onwards, however, they could be found in the majority of specimens, although they were only classed as numerous in three. This designation implied that as many as 6 or 7 osteoclasts could be found in a given transverse section of an implant. Giant cells were classified as osteoclasts only when they lay in lacunae in the bone, or when they were very closely applied to the surface of the bone. Occasionally giant cells were seen associated with trauma to the panniculus carnosus muscle. However, these seldom gave rise to confusion with the cells adjacent to bone.

The widening of the Haversian and Volkmann canals (Fig. 7) which was noted in this study from 35 days onwards was always accompanied by the presence within them of loose connective tissue. Blood vessels were sometimes quite obvious within these islands of tissue. In other cases, however, it was difficult to be certain whether apparent spaces in the tissue were of vascular origin, or merely represented loose fibrous tissue.

In the later specimens, in spite of considerable widening of the canals containing connective tissue, no osteoclasts were seen in relation to the bone in this site. This fact of course does not preclude the widening of the canals by osteoclastic resorption.

Homologous series.—The cellular reaction in the vicinity of homografts was indistinguishable from that around autografts during the first 4 days. By the 7th day, however, there was an absolute increase in the number of cells round the homografts. By comparison the tissue in the vicinity of autografts showed a diminution in the number of cells at this stage. The cell population round the homografts at 7 days consisted predominantly of lymphocytes, with occasional plasma cells and eosinophils. Mast cells were in excess of normal and the fibroblasts appeared relatively plumper than those round autografts.

The cellularity increased, reaching a maximum around the 12th day (Fig. 11). Lymphocytes were always the predominant cell and mast cells were usually in excess of the numbers seen round autografts. Plasma cells and eosinophils were occasionally seen but their presence was by no means invariable. Moderate numbers of cells were sometimes present which were interpreted as being immature plasma cells. They were rather smaller than mature plasma cells and usually showed a central nucleus. In other respects, however, they resembled plasma cells. Fibroblasts tended to be a little plumper than those seen round autografts at this stage, and the quantity of collagen was a little less.

On the 14th day the appearances were essentially similar although there was a very slight over-all diminution in cellularity. On the 35th day, while the cellularity of the host tissues round the implants was very much less, there were still occasional small collections of lymphocytes. Mast cells remained in excess of normal and there were a few mature plasma cells.

Small clumps of lymphocytes persisted at 63 days (Fig. 4), and less regularly minute aggregations could be found at 100 and 250 days. In all the late homografts the slight excess of mast cells persisted (Fig. 12), and occasional plasma cells were seen.

The tissues growing into the concavities of the homografts differed in several respects from those growing in similar sites in autografts. In the first place growth into the hollow centre of the implants appeared much slower in homografts than autografts. Because the histological sections of the implants and adjacent tissues were cut transversely, it was not possible to measure the distance which the granulation tissue had grown down the hollow tubes at a given time. The slowness of this growth in homografts was thus not appreciated until a considerable number of the later specimens had unexpectedly been found empty. At 35 days for instance it was noted that approximately half of the transverse sections of homografts showed an empty medullary cavity. Considering that the length of the hollow tube was never more than 7·5 mm. from either end this rate of growth was somewhat surprising. It compared unfavourably with that seen in the 35 day autografts, in which every one of a considerable number of sections showed the medullary cavity to be full of vascular osteogenic tissue. This slowness of growth was even more definite in the later homografts. At 250 days for instance 6 out of 32 sections from homografts still showed an empty medullary cavity. The autografts by contrast were invariably full.

In addition to this apparent retardation of growth towards the concavities of the bone in homografts, the connective tissue which finally came to occupy these spaces was morphologically abnormal in several respects. Abnormalities were readily detectable from 35 days onwards, but were seldom more than doubtful during the earlier stages, when the fibroblasts seemed a little plumper in the vicinity of homografts than they did round autografts. In the later specimens

(Fig. 13) the collagen fibres which were present appeared to run in all directions. Large spaces were evident between the fibres, giving an over-all lacy appearance (Fig. 4, 14). Some of the fibres showed a peculiar " beaded " appearance (Fig. 8, 14). Many of the fibroblasts appeared a little plump and stained rather more darkly than normal (Fig. 12). Blood vessels frequently showed hyaline thickening of their walls with a consequent relative reduction in cellularity (Fig. 15). The vessels involved in this change were all capillaries, although their calibre varied. Scarcely any conversion of new vessels into arterioles and venules was evident in homografts during the course of the experiment.

Remodelling of the homologous implant, even at 250 days, was negligible (Fig. 8). While giant cells morphologically resembling osteoclasts were visible in a few of the earlier specimens, they were infrequent by comparison with the numbers seen round autologous implants. Similarly, the widening of the bone canals seen in autografts in this series from 35 days onwards was not seen at all in homografts.

The differences in host reaction between autografts and homografts were usually clearly defined after the end of the first week, the main points being summarised as follows :—

(1) The maximum inflammatory cell infiltration round autografts occurred on the third to fourth day, a rapid diminution in the cellularity occurring subsequently. In homografts the maximum inflammatory response was seen about the 12th day, and the subsequent diminution was relatively protracted.

(2) The cytology of the homograft reaction was characterised chiefly by lymphocytes, and also to a lesser extent by mast cells and plasma cells. While all these cells could be seen round autografts during the first few days, they were not particularly numerous, the cytology being predominantly polymorph.

(3) The growth of connective tissue into the concavities of the bone implants appeared to be slower in homografts.

(4) Connective tissue occupying the concavities of homografts showed morphological abnormalities of the collagen, fibroblasts and blood vessels.

(5) Remodelling of the original implant was negligible in homografts as compared with autografts.

DISCUSSION

The prolonged survival of homologous osteocytes is not surprising, since by reason of the structure of bone, the osteocytes are virtually situated within a diffusion chamber. The " pores " within it, *i.e.* the canaliculi, are too small to allow the host inflammatory cells to gain access to the osteocytes. Because of the lack of contact between graft and host cells, the osteocytes are not destroyed at the height of the inflammatory reaction (Algire, Weaver and Prehn, 1957).

Somewhat more difficult to explain are the mode of nutrition of the surviving osteocytes and the reasons for the slightly greater proportion of osteocytes surviving in autografts than in homografts.

During the initial period after implantation the osteocytes no doubt depended on the tissue fluids of the host for their nutrition. Tissue fluids surrounded the outer circumference of the implant, and to some extent were seen to occupy the hollow centres of the bony tubes. Because the fluid in the latter site was some distance from the circulation, it was probably relatively stagnant compared with

the fluid outside the implant. Therefore the nutrition available from this source to the innermost osteocytes of the implants was relatively limited. It did appear, however, that some prolongation of survival time was effected, since osteocytes were identifiable near the inner circumference up to the 14th day. By contrast, *in vitro* experiments where the cells were deprived of exogenous nutrition showed complete degeneration of all osteocytes by the 4th day.

The cells near the outer circumference of the implants were situated more favourably in 2 respects: the tissue fluids bathing the surface were almost certainly less stagnant than those in the medullary cavity of the implant, and revascularization of this type of implant commenced from the outer surface (Zeiss *et al.* 1960).

In view of these advantages it was not surprising to find that at 35 days and subsequently, all the surviving osteocytes in both types of implant were contained in a band running round the outer circumference. It is interesting to note that the depth of this band was never more than 300 μ, and was usually considerably less. Experiments on autologous thyroid implants in rats have indicated that the tissue fluids are capable of nourishing thyroid epithelial cells up to 300 μ from the surface of an avascular specimen (Dempster and Doniach, 1955). On the other hand, Ham (1953) states that the canalicular mechanism in dogs can maintain the life of osteocytes over a distance not greater than 200 μ.

The part played by revascularization in facilitating osteocyte survival is difficult to separate from the part played by the surrounding tissue fluids. However, the invariable restriction of the surviving cells to a zone probably capable of receiving the greater part of its nutrition from the surrounding fluids, suggests that plasmatic circulation was of major significance in the circumstances of this particular experiment. If the early vascular supply had been of major functional importance, one might have expected to see islands of cells surviving indefinitely near the inner circumference, since injection studies on similar implants showed occasional vessels penetrating the whole thickness of the bone as early as the 2nd day (Zeiss *et al.* 1960). Similarly the apparent lack of histological correlation between vascular supply and cell survival in individual cases, seemed to favour the tissue fluids as the main source of nutrition to the implant, at least in those cases in which the blood supply was still incomplete.

It did appear possible, however, that early revascularization might prolong the survival of a few cells whose nutrition was otherwise precarious, *e.g.* because of their distance from the surface. Differences in the proportions of osteocytes surviving in autografts and homografts became obvious early in the experiment (Fig. 3), at a time when there were significant differences in vascularity between the two types of implant (Zeiss *et al.* 1960). It is tempting to postulate that the differences in cell survival between autografts and homografts were governed by variations in the rate and extent of revascularization. It will be noted in Fig. 3 that the proportion of osteocytes surviving in the homografts fell progressively during the first 35 days, while in autografts there was little change after the 12th day. Considering this finding in the light of vascular injection studies (Zeiss *et al.* 1960) this behaviour of homografts could well be explained by: the diminution in vascularity at the time of the homograft reaction, and the continued rearrangement of the vascular pattern in the granulation tissue round homografts. The outcome of both these phenomena was a progressive reduction in the vascularity of the granulation tissue, which was reflected in the vascularity of the

bone itself (Zeiss *et al.* 1960). The fact that the percentage of osteocytes surviving in homografts was similar at 100 and 250 days (16 and 17 per cent respectively) in spite of progressive reduction in the vascularity of the bone and adjacent tissues does not necessarily detract from this argument. It is possible that these osteocytes were sufficiently near the surface to derive their nutrition from the tissue fluids outside the implant irrespective of any changes in the vascular pattern.

The evidence from this experiment thus suggested that tissue fluids were capable of prolonging the life of the osteocytes in the implants. It seemed that in the absence of optimal conditions—which were presumed to obtain within not more than 300 μ of the outer circumference—the extent of such prolongation was limited. The cells within about 300 μ of the outer circumference soon came to possess the added advantage of a blood supply, and this was sufficiently better in autografts than in homografts to explain the significant differences between them as regards osteocyte survival. Those osteocytes near the inner circumference of the implants showed some prolongation of life (compared with *in vitro* experiments) which was ascribed to sluggish plasmatic circulation in their vicinity. Effective revascularization of this inner zone occurred too late, however, to allow indefinite survival of these cells.

As far as could be judged from this experiment it appeared likely that the proportion of osteocytes surviving in bone grafts would bear an inverse relationship to the thickness of the implant. In thick grafts, any comment on cell survival would be of little value without reference to the proximity of the histological block to the surface.

It seemed doubtful whether there was any quantitative relationship between the extent of osteogenesis and the percentage of osteocytes surviving in the implants. Absolutely no correlation was seen in individual cases. Over the whole series the differences between the percentages of osteocytes surviving in autografts and homografts were relatively small compared with the gross differences in osteogenesis (Fig. 3). The part played by the implant osteocytes in stimulating the formation of new bone remains uncertain, but the various experiments in which dead bone has been used (see De Bruyn and Kabisch, 1955) indicate that osteogenesis is greatly impaired if the graft cells do not survive. Taking these latter findings in conjunction with the present experiment, it would seem that the osteocytes of the implant may well stimulate osteogenesis in their vicinity, but the stimulus bears no apparent quantitative relationship to the ultimate extent of new bone formation.

In view of the anatomical structure of bone it seems highly improbable that the osteocytes contribute to osteogenesis by their proliferative activity, and besides this, these cells have never been observed to undergo mitosis (Pritchard, 1956). In those autografts examined 35 days after implantation the hollow centres of the tubes were largely filled with young bone. At this time the osteocytes of the implant which were nearest to the new bone were entirely dead.

The presence of other tissues in the implant (*e.g.* marrow, endosteum), which could have given rise to metaplastic bone, could not always be entirely excluded. However, in preparing the implants every effort was made to remove the soft tissues, and subsequent histological examination of the early implants suggested that this end had been achieved in the majority of cases.

Relatively little very early bone formation was seen in this series by comparison with the results of Chalmers (1959). However, the extent to which the

soft tissues had been removed from his grafts is not entirely clear, and it seems possible that inadvertently included endosteum or marrow could have given rise to metaplastic bone. On the other hand, the implants in this series were largely devoid of non-osseous tissue, a state of affairs which could be expected to retard the commencement of osteogenesis. Indeed, the later onset of osteogenesis in this experiment, as compared with that of Chalmers, suggests that the soft tissues had in fact been fairly thoroughly removed in preparing the implants.

In this series the new bone formed in association with the grafts appeared to arise as a result of metaplasia in the host tissues, since bone was not formed in the absence of granulation tissue. The latter appeared to grow down the bony tubes from both ends, a method of growth implying its origin from host connective tissues rather than from any cells included in the implant. Osteogenesis seemed to occur readily in the loose vascular tissue which came to fill the concavities of the graft, but was relatively insignificant in the denser tissue surrounding the convexities. It appeared that the predilection of the young bone for the hollow centres of the tubes and the larger surface concavities was probably related to the presence in these sites of a more suitable precursor tissue. Even where a large quantity of new bone was formed, it was more or less restricted to the concavities, the quantity of bone formed in relation to the convexities being negligible by comparison. While it is true that a large proportion of the convex outer circumference of the autologous implants ultimately came to be replaced by new bone, this was part of the later slow remodelling process, and was not to be compared with the rapid deposition of new bone seen in the early days after implantation. The reasons for the preferential conversion of granulation tissue to bone in the sites mentioned remain uncertain, but 3 factors—possibly interdependent—could conceivably contribute

(1) Relative lack of compression as compared with the tissues round the convexities.

(2) Greater quantity of ground substance relative to the number of cells in the concavities.

(3) The better blood supply offered by the granulation tissue in the concavities. This is confirmed by injection studies (Zeiss *et al.*, 1960).

The distribution of new bone in this experiment suggests one possible explanation for the fact that better clinical results are obtained with medullary bone or chips than with large grafts (Annotation, 1957). The multiple tiny grafts presumably offer more spaces for the free development of readily ossifiable granulation tissue.

In addition to the fact that the presence of spaces in the graft appeared to facilitate osteogenesis, the autograft was apparently able to confer some osteogenic stimulus which was either absent or not effective in the homograft. Several features of the present experiment warrant consideration in this connection, although the part played by each must of necessity remain speculative. In the first place the filling of the concavities in the implant was relatively slower in the homograft, and the ingrowth of granulation tissue was an invariable prerequisite for osteogenesis. Probably of greater importance though in this connection was the structural abnormality of the tissue which ultimately came to fill the spaces. This could well have rendered it unsuitable for osteogenesis. The coarse lattice-like connective tissue with its abnormal fibres and cells often

showed aggregations of granular calcified material, but true ossification was insignificant compared with the quantity of new bone seen in the normal granulation tissue round autografts. While it is difficult at this stage to supply conclusive proof that the diminished osteogenesis resulted from abnormality of the precursor tissue, the existence of an analogous state of affairs in Vitamin C deficiency (Cameron, 1952 ; Bourne, 1956) lends some support to this contention. The deficient osteogenesis in scurvy is attributed to the abnormal formation of the connective tissue which precedes bone formation. It is interesting to note that aggregations of calcified material similar to those seen round homografts are also reported in Vitamin C deficiency (Cameron, 1952).

There was no reason to think that the inflammatory cell infiltration exerted any specific inhibitory effect on osteogenesis. While it is true that almost no osteogenesis was evident at the time of the maximal cellular infiltration, it must also be remembered that very little young connective tissue was present at this time in the concavities round the homografts. By contrast the autografts at this stage usually contained copious granulation tissue which appeared to be an essential prerequisite for osteogenesis. It was also noted in homografts that the later diminution in the numbers of inflammatory cells was not accompanied by a parallel increase in osteogenesis, even when the medullary cavities contained connective tissue. Such an increase might have been expected had the inflammatory cells exerted an inhibitory effect on osteogenesis.

While very little is known about the nature of the osteogenic stimulus presumably exerted by the graft osteocytes, there is no evidence to suggest that it is individual specific. One wonders whether osteogenesis might have occurred round homografts, had it been possible in some way to induce the development of normal granulation tissue instead of the material which did in fact occupy the concavities. The specificity of this osteogenic stimulus is being further investigated at the present time.

It appeared that the abnormal connective tissue seen round the homografts was a direct outcome of the presence of the homologous bone, since it was consistently absent round autografts. It could well have represented another response to the same stimulus which evoked the inflammatory cell infiltration. If both these aspects of the response to homologous tissue do in fact result from the same stimulus, it seems reasonable to regard both phenomena as components of the homograft reaction. If the latter term is used in this broad sense, impairment of osteogenesis round homografts can reasonably be ascribed to the effects of the homograft reaction. On the other hand, if the term homograft reaction is used more conventionally to refer to the inflammatory cell infiltration, there is no evidence that impaired osteogenesis can be ascribed to its presence.

The reason for the reconstruction of the autologous implant is obscure. While much of the bone was removed by osteoclastic resorption, copious quantities of new bone were added, and the sum total of both activities was a structure reminiscent of the normal rat tibia. The conversion of the early haemopoietic marrow to fatty marrow suggested a recapitulation of the events occurring during normal development. The approximation of the reconstructed graft to the original bone was all the more surprising in view of the fact that the graft was never at any stage functioning mechanically as normal bone. Perhaps such a reconstruction might have been expected in a graft inserted in the skeleton, but the significance of such changes occurring in a suprapannicular implant remains a mystery.

The relative absence of osteoclastic activity round the homografts was interpreted as representing merely one aspect of what appeared to be a general reluctance on the part of the host tissues to approach the homologous implant. This reluctance was also indicated by the relatively slow growth of the connective tissue into the hollow centre of the homograft, and likewise by the much slower revascularization of the implant as compared with the autograft (Zeiss *et al.* 1960). The fact that these later aspects of the homograft reaction have received scant attention in the past is no doubt related to the predominant use of soft tissues in experimental grafting. Bone possesses two features, the combination of which offers an excellent opportunity for studying the effects of prolonged antigenic stimulation by a homologous graft. In the first place a proportion of its component cells survive for a long period, in spite of the fact that they are able to excite a fairly typical inflammatory reaction towards the end of the second week. Bone also possesses the advantage that it is not necessarily rejected from the body in the event of death (Baker, 1959), *i.e.* the death of the inner circumference of the bony tubes in this series should not necessarily prejudice the acceptance of the implants. It is thus possible for the foreign osteocytes to survive and function over a long period of time—longer than would be possible in soft tissues without having recourse to one of the methods for prolonging the survival time of homografts (Medawar, 1957).

While some of the phenomena seen in connection with the late homograft reaction in this experiment seemed to be explicable, others remained difficult to account for. No satisfactory explanation was available for the relative reluctance of the host tissues to approach the homograft. Likewise, the morphological peculiarities of the connective tissue adjacent to the homografts were difficult to interpret. The hyaline thickening of the blood vessel walls could have been analogous to that sometimes encountered in areas of chronic inflammation, although the vascular changes often seemed rather advanced relative to the severity of the inflammation. The possibility was entertained that this hyaline change was akin to amyloidosis, *i.e.* it might have resulted from prolonged stimulation of the antibody forming mechanism (Marshall, 1956). The Congo red stain was negative, however. Even making allowances for the difficulty in staining some types of amyloid, it seemed doubtful whether prolonged stimulation of the antibody-forming mechanism associated with transplantation immunity could necessarily be held to have the same effects as prolonged stimulation of the mechanism associated with bacterial immunity.

The lymphocytes and plasma cells seen around the homografts at 35 days and subsequently were always present in much scantier numbers than at 14 days, the infiltration of these cells reaching a maximum in the early stages after implantation and subsequently diminishing. The numbers seen at 100 and 250 days were sufficiently small to have excited little comment, had it not been for the presence of the more spectacular infiltration in the earlier specimens with which they were being compared. The reason for this diminution remains uncertain. Differences in the proportions of surviving homologous osteocytes—which presumably excited the reaction—were not sufficiently great to explain the differences in cellular infiltration on the basis of quantitative stimulation. In theory two possible explanations might be suggested :—(1) an exhaustion of the recipient's reticuloendothelial system. From the results of the experiments by Thomas, Murray and Couch (1957), however this appears to be untenable. (2) a change or decrease

in the antigenicity of the grafted cells. This view would accord with Woodruff's theory of adaptation (Woodruff, 1959).

The increase in the numbers of mast cells round the homografts could reasonably be explained as a concomitant of fibroblastic proliferation and chronic inflammation (Janes and McDonald, 1948; Bensley, 1950; Wichmann, 1955), particularly as the implants occupied a site normally rich in mast cells. There was no reason to think that they subserved a specific function in connection with the homograft reaction, although of course such a possibility could not entirely be excluded. While there was nothing in this experiment to suggest such a specific function, it is interesting to note that the rapid proliferation of the mast cells of the rat during the first 19 days of life (Webb, 1935) corresponds approximately with the period during which it is possible to produce immunological tolerance in this animal (Woodruff and Simpson, 1955). While this association could well be fortuitous, it probably warrants further investigation.

SUMMARY

Tubular portions of autologous and homologous cortical bone were implanted suprapannicularly in rats, and were subsequently excised at various times after insertion. On an average, 6 specimens per day were examined histologically 2, 3, 4, 7, 10, 12, 14, 35, 63, 100 and 250 days after implantation.

A proportion of the osteocytes survived up to 250 days near the outer surface of both autografts and homografts. Correlating these results with vascular injection studies (Zeiss *et al.* 1960), the survival of osteocytes was largely attributed to plasmatic circulation, although the slight superiority of autografts in this respect was explicable by the readier establishment of a blood supply.

Osteogenesis was copious in all autografts at 35 days. By contrast it remained negligible or absent in homografts at 250 days. There was no quantitative relationship between osteocyte survival and osteogenesis, although it seemed likely that a qualitative relationship existed, *i.e.* the presence of living osteocytes was probably necessary in these implants if osteogenesis was to occur. There was no reason to think that the osteogenic stimulus was individual specific. It appeared much more likely that the failure of new bone formation round homografts was due to the presence of an abnormal precursor tissue.

Because the homologous osteocytes survived, it was presumed that they continued to exert an antigenic stimulus over a longer period of time than is usually possible with homologous cells. In addition to a predominantly lymphocytic inflammatory cell infiltration about the end of the first fortnight, the granulation tissue associated with the homografts, particularly that in the hollow medullary cavities, showed gross morphological abnormalities of both collagen, fibroblasts and blood vessels. This tissue could well have been unsuitable for ossification.

The rate of growth of granulation tissue was strikingly slower round homografts than in relation to autografts. This feature, taken in conjunction with the lack of osteoclastic differentiation and resorption, and also the slower vascularization described elsewhere (Zeiss *et al.* 1960), was interpreted as a reluctance on the part of the host reparative tissues to approach the homologous implant. The homografts also showed an excess of mast cells in their vicinity, but the specificity of this reaction remains doubtful.

It was noted round the autografts that ossification occurred preferentially in

the loose young granulation tissue in the concavities of the bony implant. It was rare in relation to the convexities. This might explain the better results obtained clinically with medullary and chip grafts, which offer relatively more abundant spaces in which this preosseous tissue can develop.

The late remodelling and replacement of the original autologous implants was not seen in the homologous implants. The latter appeared to retain more or less their original contours for the duration of the experiment.

We wish to acknowledge the assistance of Mr. G. F. S. Spears, M.Sc., Statistician, Otago Medical School, for analysis of these results. We are grateful to Mr. G. Bills and Mr. F. Aitken for technical assistance. The photomicrographs were prepared by Mr. F. H. Knight and the diagrams by Miss L. Duncan. Part of this work was supported by a grant from the N.Z. Crippled Children's Society.

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THE HIGH MOLECULAR WEIGHT COMPONENTS OF SPUTUM

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Received for publication November 30, 1959

THE healthy human bronchial tree is estimated by Policard and Galy (1945) to produce about 100 ml. of mucus in 24 hr., which is normally swallowed together with the secretions of the upper respiratory tract and bucco-pharynx. In certain respiratory diseases, notably chronic bronchitis and asthma, this mechanism becomes deranged and an alteration in the character of the secretion occurs together with a tendency towards excessive formation. This excessive and altered secretion is of significance in chronic bronchitis, as it causes obstruction of the smaller air-passages (Oswald, 1958). Similarly, the obstructive role of the viscid mucus in intrinsic bronchial asthma is a factor of considerable importance in the aetiology of this syndrome (Bukantz, 1958). A prominent clinical feature of chronic bronchitis and asthma is the expectoration of the intra-bronchial accumulation of mucus as sputum. Despite the likely role played by the mucous coagulum of sputum in such chronic respiratory diseases, relatively little work has been carried out on the ground substance of sputum since the investigations of Muller (1896 ; 1901).

In a recent paper (Brogan, 1959), the carbohydrate complexes of pooled sputum were examined. Two mucopolysaccharide fractions, similar in type to the blood-group substances, and a mucoprotein, which had some properties in common with the urinary mucoprotein described by Tamm and Horsfall (1952), were identified. Similar findings on the mucopolysaccharide fraction of sputum were obtained previously by Bukantz and Berns (1958) who also showed, serologically, that this fraction possessed blood-group activity. Both Bukantz and Berns (1958) and Brogan (1959) isolated the carbohydrate complexes of sputum by digestion with pepsin of sputum which had been precipitated with ethanol or dried with acetone. Although digestion with a proteolytic enzyme is an adequate method of isolating the carbohydrate complexes, which amount to less than 50 per cent of the total solids of pooled sputum, such a method decomposes all the protein complexes.

An investigation was therefore carried out with the object of ascertaining the nature of the other high-molecular-weight components of sputum. The purpose of this work was to attempt to elucidate further the nature of the gel composing the mucous ground-substance of sputum. Methods of rendering the mucous ground-substance of sputum soluble in water by shaking with ballotini or by the high-speed stirring technique, described by Marmion, Curtain and Pye (1953), were found to be unsatisfactory because foaming of the mucin occurred together with considerable denaturation of the components in the air-liquid interface of the foam. A means was sought, therefore, by which mechanical

shock could be applied to the mucous coagulum of sputum without causing it to foam. Ultrasonic vibration was found to fulfil this requirement.

The present paper describes a method of homogenizing sputum, using ultrasonic vibration, which renders the high-molecular-weight complexes, forming the coagulum, soluble in water whilst largely preserving the identity of the major components.

MATERIALS AND METHODS

Sputum.—Sputum was collected from patients who were suffering from long-standing respiratory diseases such as chronic bronchitis and asthma. Only specimens which would be assessed clinically as " mucoid " or faintly " mucopurulent " (Chamberlain, 1957), were retained for use. Each mucous coagulum was inspected and visible foreign bodies were removed. The mucous coagula were washed in several changes of water and were pooled together in a beaker. The volume of the pooled mucin was measured before transferring it to the metal cup of the ultra-sonic vibrator.

Analytical methods.—The methods of estimating total nitrogen, organic and inorganic phosphorus, reducing sugars, amino sugars, and pH have been described previously and were used without change. Similarly, the methods used for the paper partition chromatography and for the colorimetric and spectrophotometric measurements were the same as those used in the previous work (Brogan, 1959).

Electrophoresis.—Tiselius electrophoresis was carried out with a Perkin-Elmer apparatus.

Paper electrophoresis was carried out by a method closely similar to that described by Franglen (1953). Platinum electrodes were used in the electrophoresis apparatus and 0·05 M-sodium diethylbarbiturate-HCl buffer, pH 8·2, was employed. The electrophoresis was carried out for 12 hr. at a p.d. of 120 v. using Whatman No. 100 paper. Electrophoreses of sputum and of ultrasonically homogenized sputum were carried out simultaneously with that of normal human serum. Bromophenol blue was used to stain the bands.

Immuno-electrophoresis was carried out by a method based on that of Grabar and Williams (1955). The agar gel plates were prepared by mixing equal volumes of filtered 3 per cent agar and 0·1 M-sodium diethylbarbiturate-HCl buffer, pH 8·2, thus giving a final agar concentration of 1·5 per cent, the ionic strength of the block being 0·05. The dimensions of the agar slabs were 17 × 8 × 0·5 cm. and the wells for the antigens were each 2 × 0·3 cm., and were set with their inner edges approximately 1 cm. from the central channel. The potential gradient across the gel was 4 v. cm.$^{-1}$ and the electrophoresis was carried out for 5 hr. Rabbit anti-human serum was used as indicator in the central channel and normal human serum, as a 10 per cent (v/v) solution in 0·05 M-sodium diethylbarbiturate-HCl buffer, pH 8·2, was used as the control antigen. After the electrophoretic runs, the agar slabs were kept at room temperature in an atmosphere saturated with water vapour so that precipitin band formation could take place.

Ultra-sonic homogenization of sputum.—The following procedure proved most satisfactory for homogenizing a pool of sputum. Sputum (50 ml.) was poured into the metal cup of a Mullard Ultra-sonic Generator, E 6590 A, and the cup and its contents were allowed to come to 0°. The sputum was then subjected to ultra-sonic vibration at 20 k. cyc./sec. for 2 hr. A shorter period of time than 2 hr. always resulted in incomplete dissolution of the coagulum. The resulting solution, which had a greyish opalescence, was centrifuged at 0° at 1000 **g** to remove insoluble material. The supernatant solution was dialysed for 17 days against water at 0° and was then centrifuged at 15,000 **g** to remove a small amount of insoluble material. The resulting solution was then dried from the frozen state. A voluminous white powder (625 mg.) resulted.

The combined residues from the ultra-sonic homogenization were then washed several times with water and dried *in vacuo*. A grey-white powder (55 mg.) resulted. Microscopy showed that this residue was largely organised, consisting of the remains of leucocytes, epithelial cells and bacteria. There was also some amorphous basophilic material present.

The total yield of material, computed on a w/v basis, was 1·36 per cent of the parent sputum, being made up of 1·25 per cent of water-soluble high-molecular-weight complexes and 0·11 per cent of insoluble residue. This compares with an overall yield of 1·5 per cent for acetone-dried sputum (Brogan, 1959).

RESULTS

Properties of the ultra-sonically homogenized sputum.

Chemical properties.—The ultra-sonically homogenized sputum dissolved in water to give a solution of neutral pH. Addition of dil. HCl to pH 6·0 caused precipitation but continued addition of acid to 0·5 N-HCl caused the precipitate to re-dissolve. If NaCl was added to the solution to I = 0·005, precipitation did not occur with dil. HCl until pH 4·6 was reached. No precipitation occurred when NaCl was added, to a concentration of 0·58 M-NaCl, to a neutral aqueous solution of the ultrasonically homogenized sputum, but precipitation occurred when equal volumes of reagents that normally precipitate proteins were added to such solutions.

TABLE I.—*Comparison of the Composition of Ultra-sonically Homogenized Sputum and Acetone-dried Sputum.*

Analyses of the preparations were carried out on samples which were dried at 56° for 1 hr. The analytical results are expressed as a percentage of this dry weight. For methods of analysis, see text.

	Ultra-sonically homogenized sputum	Acetone-dried sputum
Yield (per cent)*	1·25	1·50
Total N (per cent)	8·8	10·4
Amino sugars (per cent)	9·3	10·8
Reducing sugars (per cent)	17·4	18·7
Total P (per cent)	0·08	0·62
Inorganic P (per cent)	0·08	—
Organic P (per cent)	Nil	—
Ratio amino sugars/total N	1·1	1·0

* The percentage yield was computed on a w/v basis with the original sputum.

In Table I, the analysis of ultra-sonically homogenized sputum is compared with that of acetone-dried sputum. It may be seen that the reducing-sugar content and that the ratio of amino sugars : total nitrogen are comparable with those of acetone-dried sputum. The total phosphorus content of the ultra-sonically homogenized sputum, however, is lower than that of acetone-dried sputum, and is made up wholly of inorganic phosphorus.

Physical properties.—In Fig. 1, the ultra-violet absorption spectrum of a 0·025 per cent solution of the ultra-sonically homogenized sputum in 0·85 per cent NaCl is compared with that of a comparable solution of the mucoprotein prepared from acetone-dried sputum (Brogan, 1959). This latter complex contained 0·74 per cent of organic phosphorus. It may be seen that the λ_{max} for the ultra-sonically homogenized sputum is 278 mμ compared with the λ_{max} for the sputum mucoprotein, which is 259 mμ. The total nitrogen contents of the ultra-sonically homogenized sputum and of the mucoprotein prepared from acetone-dried sputum are 8·8 per cent and 8·7 per cent respectively. The ultra-violet absorption spectrum together with the absence of organic phosphorus in this complex suggests that this preparation of the high-molecular-weight components of sputum probably does not contain nucleic acids. This is in contrast with the sputum mucoprotein prepared from acetone-dried sputum, as both the ultra-

violet absorption spectrum of this complex and its content of organic phosphorus indicate that the substance contains nucleic acids.

Tiselius electrophoresis of the ultra-sonically homogenized sputum was carried out in a 0·02 M-Na_2HPO_4-NaH_2PO_4 buffer, pH 7·4, containing 0·15 M-NaCl, for 75 min. at 105 v and 20mA. In Fig. 2, the ascending electrophoretic pattern of the ultra-sonically homogenized sputum is compared with that of normal human serum, the electrophoresis of which was carried out under the same conditions. It may be seen that the ultra-sonically homogenized sputum is composed of five components. The two lesser components, 1 and 5, account for approximately 13 per cent and 10 per cent of the total complex respectively. Components 2 and 3 together account for approximately 43 per cent of the complex, and com-

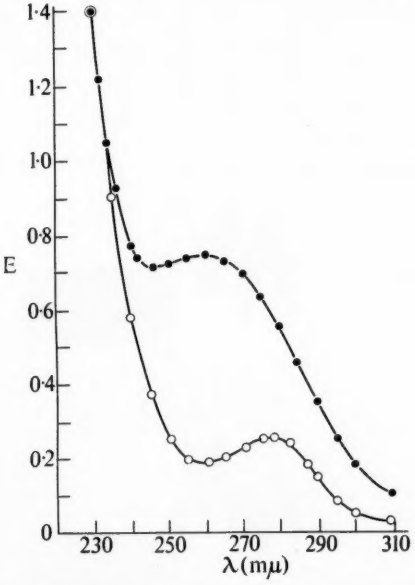

FIG. 1.—Ultraviolet-absorption-spectra curves of 0·025 per cent solutions of ultrasonically homogenized sputum (○) and of the sputum mucoprotein (●), prepared by digesting acetone-dried sputum with pepsin. Both complexes were dissolved in aq. 0·85 per cent NaCl. The λ_{max} for the ultrasonically homogenized sputum was 278 mμ and the λ_{max} for the sputum mucoprotein was 259 mμ.

ponent 4 for approximately 34 per cent of the complex. These figures are calculated by the method of Tiselius and Kabat (1939). It may be seen from Fig. 2 that component 4 of the ultra-sonically homogenized sputum migrates at the same speed as normal human serum-albumin.

Paper electrophoresis of the ultra-sonically homogenized sputum was carried out on a 1 per cent solution of the material in 0·05 M-sodium diethylbarbiturate buffer, pH 8·2, for 12 hr. at a p.d. of 120 v. Paper electrophoresis of normal human serum was carried out simultaneously as a control. The ultra-sonically homogenized sputum was resolved into two bands, one of which moved at the

same speed as the albumin of the human serum. The other band remained at the origin. It is the experience of the author that mucopolysaccharides and proteins conjugated with polysaccharides, such as the mucoproteins which can be isolated

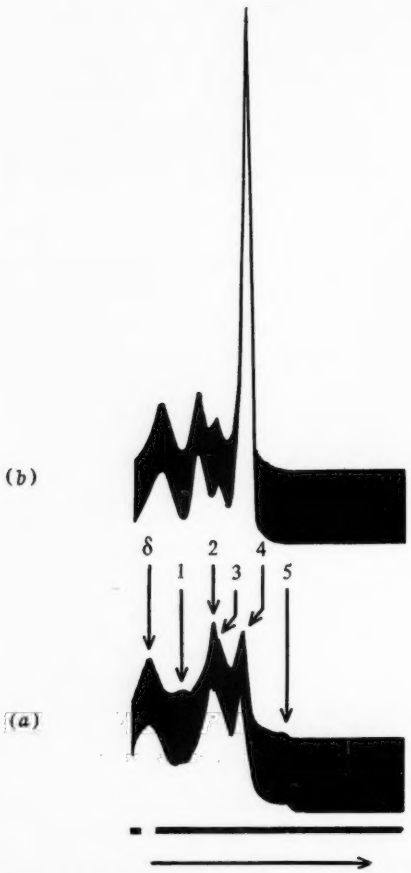

FIG. 2.—(*a*) Ascending Tiselius electrophoretic pattern of ultrasonically homogenized sputum showing the 5 components of this complex. (*b*) Ascending Tiselius electrophoretic pattern of normal human serum. Both the electrophoretic runs were carried out in 0·02M-Na_2HPO_4-NaH_2PO_4 buffer, pH 7·4, containing 0·15M-NaCl, at 100 v. and 20 mA. Both patterns were scanned at 75 min. The concentration of the ultrasonically homogenized sputum was 2 per cent (w/v) and that of the normal human serum was 25 per cent by vol. in the above phosphate buffer, the solution being dialysed against the buffer for 24 hr.

from sputum and urine, do not migrate on paper under the influence of an electric field. It may be suggested, therefore, that the component that migrates at the same speed as human serum albumin behaves as a protein in so far as it is able to move freely in an electric field on paper.

Paper electrophoresis of non-homogenized sputum, from the same pool as that from which the ultra-sonically homogenized sputum was prepared, was also carried out under the conditions described above. The purpose of this run was to ascertain whether the fraction which is present in ultra-sonically homogenized sputum and moves at the same speed as human serum albumin would be free to migrate in an electric field on paper from the native sputum coagulum. Only one band at the origin, however, was obtained : no change was observed if the time of electrophoresis was extended to 48 hr. This fraction, therefore, is

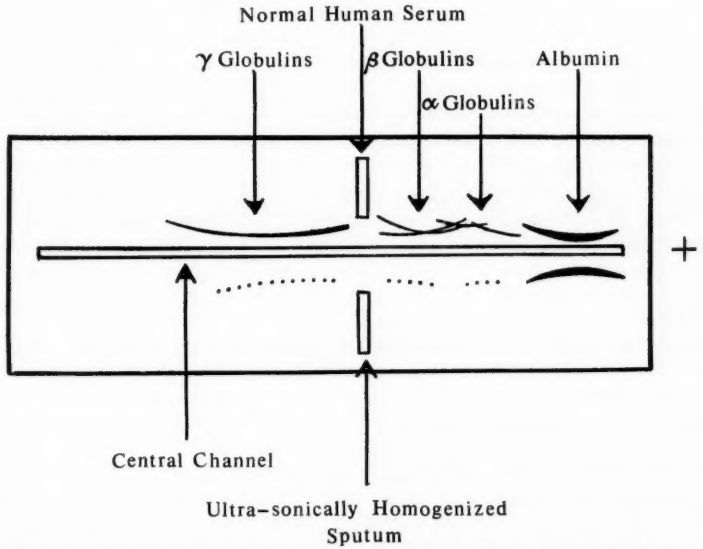

FIG. 3.—Immuno-electrophoresis of ultrasonically homogenized sputum, showing the precipitin zone corresponding to human serum albumin. The dotted lines indicate the faint precipitin zones, corresponding to the α, β and γ globulins which appeared after the agar-gel slab had been allowed to stand for 3 days at room temperature. The immuno-electrophoresis was carried out for 5 hr. at a potential gradient of 4 v cm.$^{-1}$. Normal human serum was used as control antigen and rabbit anti-serum to normal human serum was used, in the central channel, as indicator of precipitin formation.

apparently in a state in the parent sputum coagulum in which it is precluded from free movement in an electric field.

Immuno-electrophoresis was carried out on a 1 per cent solution of the ultra-sonically homogenized sputum in 0·05 M-sodium diethylbarbiturate buffer, pH 8·2. Normal human serum, diluted to a concentration of 10 per cent with the same buffer was used as a control and rabbit anti-human serum was used as an indicator of precipitin formation. The run was carried out for 5 hr. Fig. 3 gives the results of the immuno-electrophoresis. An intense precipitin zone was formed against the anti-serum by a fraction of the ultra-sonically homogenized sputum. This precipitin zone corresponded in position to that formed by the albumin of the human serum control. Faint precipitin zones corresponding to the α, β and γ globulins were formed but these did not appear until the gel had been kept

at room temperature for several days. It is thus likely that the component of ultra-sonically homogenized sputum that migrates at the same speed as human serum albumin, both in the Tiselius apparatus and in the paper electrophoresis apparatus, is composed largely of human serum albumin.

Partial Separation of the Carbohydrate Components of Ultra-sonically Homogenized Sputum

As the mucoprotein component of sputum was known to be insoluble at pH 3·3, this property was used to effect the separation of the mucoprotein complex of the ultra-sonically homogenized sputum from the mucopolysaccharide fraction in the manner described below. This method, however, was able to achieve only partial separation of these carbohydrate components from the albumin complex of the ultra-sonically homogenized sputum.

Ultra-sonically homogenized sputum (250 mg.) was dissolved in 50 ml. of water, NaCl was added to I = 0·005 and the solution was adjusted to pH 4·6 with 0·05 N-HCl. The addition of NaCl to I = 0·005 was a necessary step as pan-precipitation of all the components at pH 6·0 was liable to take place if 0·05 N-HCl were added to salt-free solutions of the ultra-sonically homogenized sputum. The resulting precipitate was centrifuged, suspended in 40 ml. of water and 0·05 N-NaOH added drop-wise until the precipitate dissolved, the resulting solution being pH 7·4.

Further 0·5 N-HCl was added to the supernatant solution, which largely contained the mucopolysaccharide fraction, until pH 1·8 was reached. No further precipitation occurred. The solution was neutralized with 0·5 N-NaOH, dialysed for 7 days against water at 0° and dried from the frozen state. A voluminous white powder A (56 mg.) resulted.

NaCl was added to the solution of the precipitate to I = 0·02 and the solution was adjusted to pH 1·8 with 0·5 N-HCl. The resulting precipitate was allowed to settle overnight and was then centrifuged. It is of interest that the supernatant of this stage contained most of the albumin fraction of the ultra-sonically homogenized sputum, the preliminary additiou of NaCl to I = 0·02 apparently encouraging this partial separation. The centrifuged precipitate was resuspended in 20 ml. of water and 0·05 N-NaOH was added drop-wise to dissolve the suspension, the resulting solution being pH 7·4. 0·05 N-HCl was added to this solution until pH 3·0 was reached ; the resulting precipitate, which was largely composed of the mucoprotein component of the ultra-sonically homogenized sputum, was allowed to stand overnight. The precipitate was then centrifuged, redissolved in 20 ml. of water by the drop-wise addition of 0·05 N-NaOH to pH 7·4, and the resulting solution was dialysed against water at 0° for 7 days and then dried from the frozen state yielding a voluminous white powder B (39 mg.).

Tiselius electrophoresis of the mucopolysaccharide fraction A showed that the main component corresponded to component 2 (Fig. 2) of the Tiselius pattern of the ultra-sonically homogenized sputum, and that it was non-homogeneous. Paper chromatography of a 0·5 N-HCl hydrolysate of this fraction revealed that amino sugars, galactose, fucose and traces of mannose were present. This is in contrast with the sputum mucopolysaccharide, isolated by peptic digestion of acetone-dried sputum, as no trace of mannose was found in this substance. (Brogan, 1959).

Tiselius electrophoresis of the mucoprotein fraction B showed that the main component corresponded to component 3 (Fig. 2) of the Tiselius pattern of the ultra-sonically homogenized sputum. The electrophoresis also showed that the main component was essentially homogeneous, in contrast with that of the mucopolysaccharide fraction A. Paper chromatography of a 0·5 N-HCl hydrolysate of the mucoprotein fraction B showed that it contained amino sugars, galactose, mannose and fucose. The electrophoretic patterns of both fractions A and B showed that each contained some of the albumin fraction of the parent ultra-sonically homogenized sputum.

DISCUSSION

The mucous ground-substance of sputum may be regarded as a gel, consisting of a discontinuous phase which, to all intents, is insoluble in water, and a continuous phase consisting of water, dissolved salts and possibly colloids in solution. The method of ultra-sonically homogenizing this gel very probably depended on the breaking down of the water-insoluble complex, forming the discontinuous phase, by mechanical shock. This procedure also may have resulted in some denaturation of the components forming the gel. The method, nevertheless, made possible the conversion of the sputum coagulum into a water-soluble state. The percentage dry-weight of the colloids of this solution together with the insoluble and largely organized residue was, in total, comparable with the precentage dry-weight obtained by drying sputum with acetone, especially when the fact is taken into account that the ultra-sonically homogenized preparation was salt-free in contrast with acetone-dried sputum.

The ultra-violet absorption spectrum of the ultra-sonically homogenized sputum together with the absence of organic phosphorus in this substance suggests that nucleic acids were very possibly absent from this preparation. This is in contrast with the mucoprotein, prepared from acteone-dried sputum by enzymic digestion, which had a relatively high organic phosphorus content and which had an ultra-violet absorption spectrum suggestive of the presence of nucleic acids (Brogan, 1959). Their presence, in this complex, may well have been an artefact of the method of preparation, and it may be suggested that nucleic acids very probably do not play a role in the structure of the gel forming the mucous ground-substance of sputum. This view finds support in the work of Armstrong and White (1950), who showed that fibres giving a positive Feulgen reaction were present in " purulent " sputum but absent from the ground substance of " mucoid " sputum, only the deoxyribonucleic acids of the cell nuclei of the latter type of sputum giving a positive Feulgen reaction. This work has since been confirmed by White, Elmes and Walsh, (1954), using phase contrast and electron microscopy. These workers also showed that incubation of the mucus from " mucoid " sputum with deoxyribonuclease and Mg^{2+} had no effect on the fibre structure of " mucoid " sputum.

Tiselius electrophoresis of the ultra-sonically homogenized sputum showed that at least five components were present. One of the components, accounting for approximately 34 per cent of the total colloids, was identified with human serum albumin. This component was not free, in the original sputum, to migrate in an electric field on paper, whereas it moved freely on paper when the sputum was ultra-sonically homogenized. This albumin fraction, in the native state, is therefore probably bound in complex formation possibly with one or

other or both of the carbohydrate complexes, to form the insoluble discontinuous phase of the sputum gel. This finding is in accordance with the work of Warfvinge (1956), who has investigated, by paper electrophoresis, the sputa of a large series of patients with various lung and bronchial diseases. He reports that only a faint and sometimes totally absent fraction of protein character, which moved on paper at the same speed as human serum albumin, was normally found in the specimens of sputum. In one case, a patient suffering from the comparatively rare bronchiolar carcinoma (pulmonary adenomatosis), an intensely staining band moving at the same speed as human serum albumin was found on examination of the sputum by paper electrophoresis. This finding was so unusual that Warfvinge (1956) suggested that it might be of use in the laboratory diagnosis of bronchiolar carcinoma.

Separation of the carbohydrate components of the ultra-sonically homogenized sputum showed that these fractions corresponded with the other two major components of the parent ultra-sonically homogenized sputum, which together accounted for approximately 43 per cent of the total colloids. The main component of the mucopolysaccharide fraction was electrophoretically non-homogeneous in contrast with that of the mucoprotein fraction, which was essentially homogeneous. The acid hydrolysate of the mucopolysaccharide fraction contained traces of mannose in addition to amino sugars, galactose and fucose. In this respect, this fraction differed from the mucopolysaccharides prepared by peptic digestion of acetone-dried sputum. The presence of mannose in this fraction may be an indication of the destructive effect of ultra-sonic vibration on macromolecules, as the mannose is likely to have originated from the mucoprotein fraction of the sputum. It is of interest that Bukantz and Berns (1958) also found mannose in the mucopolysaccharide fraction they prepared from sputum, and it is noteworthy that these workers used prolonged periods of incubation with pepsin to isolate their preparations from ethanol-precipitated sputum. In most other respects, both the mucopolysaccharide fraction and the mucoprotein fraction resembled the corresponding complexes isolated previously from acetone-dried sputum (Brogan 1959). As the method of separation was unable to free either of the fractions from the albumin component of the parent ultra-sonically homogenized sputum, it was not possible to compare the analyses of these fractions with those of the preparations from acetone-dried sputum.

The other two components of ultra-sonically homogenized sputum, together accounting for approximately 23 per cent of the total colloids, were not identified. The mobility of one component, in the Tiselius apparatus, exceeded that of human serum albumin and the mobility of the other lay between that of the β globulin and that of the γ globulin fractions of human serum. In addition to these minor components, immuno-electrophoresis showed that traces of α, β and γ globulins were also present.

Since a feature of respiratory diseases, such as chronic bronchitis and asthma, is an alteration in the nature as well as the amount of bronchial secretion such that it is expectorated as sputum (Oswald, 1958), the following mechanism of change from physiological bronchial secretion to pathological sputum may be suggested. In the present state of knowledge, it is not possible to establish the composition of normal bronchial secretion, as any intubation procedure is likely, in its own right, to produce inflammation of the mucosa. The presence, however, of mucopolysaccharides and mucoproteins in the normal secretions of more readily

available epithelial surfaces such as the gut and the urinary tract indicates that these substances may largely comprise normal healthy bronchial mucus. Under the stimulus of inflammation, it is probable that an increase in the permeability of the pulmonary vascular bed takes place with the likely result that albumin, being the smallest of the plasma proteins, is the first to make its way into the extra-cellular spaces of the mucosa. It is possibly at this site that the complex constituting the water-insoluble phase of the sputum gel, is formed between the extravasated albumin and the polysaccharides of the normal bronchial secretion. With the progression of inflammation, the initial " mucoid " sputum may become purulent, the deoxyribonucleo-protein complexes, described by Armstrong and White (1950), further contributing towards the viscidity.

SUMMARY

A method has been described by which pooled sputum can be homogenized by ultra-sonic vibration.

A water-soluble complex, containing 8·8 per cent of nitrogen, was obtained. There was evidence that nucleic acids were not present in this substance. Tiselius electrophoresis showed that the preparation was composed of at least five components.

One of the major components, accounting for approximately 34 per cent of the complex, was identified with human serum albumin. The other two major components, accounting for approximately 43 per cent of the complex, were shown to be a mucopolysaccharide fraction and a mucoprotein fraction.

Evidence was obtained that the albumin fraction was possibly bound in complex formation to form the insoluble phase of the sputum gel. It was suggested that the albumin fraction may have originated from the pulmonary vascular bed as a result of inflammatory change.

The author is indebted to Dr. F. Himmelweit for both his introduction to this work and his continued interest in its progress. Grateful acknowledgements are further due to Professor W. T. J. Morgan and Professor A. Neuberger for valuable advice and suggestions.

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HISTOCHEMICAL AND BIOCHEMICAL STUDIES IN ACUTE POISONING WITH TANNIC ACID

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Received for publication December 9, 1959

THE recognition of the hepatotoxic action and carcinogenicity of tannic acid (Korpássy, 1949 and 1959 ; Korpássy and Kovács, 1949 ; Korpássy and Mosonyi, 1950) raised a number of problems among which the most important seemed to us to be the mechanism of action of this toxic agent. As a first step towards the solution of this problem we studied the changes induced in the liver of rats by a single toxic, nonlethal, dose of tannic acid.

MATERIALS AND METHODS

The experimental animals were 66 young adult white rats of both sexes, average weight 200 g. One week before the start of the experiments they were placed on a standard diet containing casein 16 per cent, ground maize 56 per cent, dextrin 13 per cent, sunflower-seed oil 11 per cent, NaCl 3 per cent and yeast powder 1 per cent. In addition they received fresh green vegetables and water *ad lib.* Nine rats served as untreated controls. Experimentally, 57 rats were divided into 9 groups. They were given subcutaneously one dose of tannic acid of 250 mg./kg. body weight from a 2·5 per cent aqueous solution. They were killed by exsanguination at 30 min. (5 rats), at 1 hr. (5 rats), at 3 hr. (5 rats), at 6 hr. (5 rats), at 12 hr. (5 rats) at 24 hr. (9 rats), at 48 hr. (9 rats), at 72 hr. (9 rats) and one week (5 rats) after the injection. All rats were immediately dissected.

Histological and histochemical procedures.—The following techniques were used :

Haematoxylin-eosin stain after Carnoy fixation ; demonstration of glycogen by periodic acid-Schiff (PAS) reaction after Carnoy fixation ; fat stain with Sudan III–IV (Herxheimer) after calcium-formol fixation on sections cut by freezing microtome; methylgreen-pyronin stain after Carnoy fixation, for ribonucleic acid ; Feulgen's reaction after Carnoy fixation, to demonstrate deoxyribonucleic acid (DNA) ; demonstration of proteins by oxidized tannin-azo dye method (Dixon, 1959) after Carnoy fixation ; Demonstration of mitochondria by Regaud's method after fixation in Champy's solution ; demonstration of succinic dehydrogenase in unfixed frozen sections by the neotetrazolium method. (Cascarano and Zweifach, 1955) ; examination of lipase activity in frozen sections (10 μ) according to the method of Gomori (1945), after fixation in Baker's chloralhydrate-formol at 4° for 24 to 30 hr. (Baker, Hew and Fishman, 1958), the substrate for the lipase was Tween 80 and the sections were incubated for 12 hr.

Biochemical methods.

The glycogen content of liver tissue was determined by the method of Good, Kramer and Somogyi (1933).

For paper-chromatography of free amino acids extracted from liver tissue, a piece of liver (0·8–1·0 g.) was homogenized with absolute ethanol and the suspension filtered ; the filtrate was evaporated to dryness and the residue taken up in 95 per cent ethanol (1 ml. for extract obtained from 1 g. liver) ; 0·1 ml. of the resulting fine suspension was applied in small portions to Whatman No. 1 paper. The solvent for the development of the chromatograms at 26° was a mixture of butanol, acetic acid and water, 4 : 1 : 4 ; the spots were visualized by spraying with a 0·2 per cent ninhydrin solution.

Paper-electrophoresis of serum proteins was made in Veronal buffer, pH 8·6, at 120 V. (paper Macheray-Nagel No. 214). The strips were stained with acid fuchsin and evaluated with a Spinco Analytrol densitometer.

RESULTS

The first histological signs of liver damage appeared 12 hr. after the injection of tannic acid : swollen parenchyma cells, contracted sinusoids, strongly acidophilic staining of the cytoplasm in some centrolobular cells and pyknosis of nuclei. These changes became more pronounced at 24 hr. and by 48 hr. extensive centrolobular necroses and severe hydropic degeneration of liver cells were seen ; at the same time numerous mitoses appeared as a sign of regeneration. At 72 hr. the same changes were still present although in a milder form. By the end of a week the necrotic areas disappeared, but a moderate degree of hydropic degeneration persisted. Apart from a few mitotic nuclei, there were many very large, frequently oval, hyperchromatic nuclei present.

The gross histological signs of hepatic damage were preceded by a decrease in the glycogen content of liver cells as early as 30 min. after the injection of tannic acid. At 1, 3 and 6 hr. glycogen was barely demonstrable in the histochemical preparations, but in the liver of the animals killed 12 hr. after the injection of tannic acid a repletion of the liver cells with glycogen at the periphery of the lobules commenced. By 48 hr. the glycogen picture—apart from the necrotic areas—was very similar to that seen in controls ; after one week complete restitution of the glycogen content occurred. At no time after an injection of tannic acid could we demonstrate an increased fat content of liver cells by Sudan-stain.

A parallelism was seen between the gross histological lesions and the ribonucleic acid (RNA) content of the liver cells : 1–6 hr. after the administration of tannic acid the RNA was seen in coarse granules within the cytoplasm ; at 12 hr. the RNA content of the cells around the central veins was much diminished. This change later became more conspicuous and at 48 hr. pyronine-staining granules could not be seen in the necrotic cells. Restitution towards normal was seen at 72 hr. and after one week the RNA content of the liver cells was normal or even greater than normal.

The changes in DNA content were not particularly characteristic in the early phases of liver damage as judged by the Feulgen reaction which was negative only in the necrotic areas. One week after the administration of tannic acid, however, the Feulgen reaction indicated an increased DNA content in many cells particularly in the cells with the enlarged hyperchromatic nuclei (Fig. 1) mentioned earlier.

The proteins stained by the method of Dixon (1959) appeared in the liver cells of normal animals as fine evenly distributed granules, all cells giving identical pictures. Very soon (30 min.) after injection of tannic acid many pale staining cells appeared (Fig. 2), especially in the central areas of the lobules where the staining for proteins became entirely negative a few hours after the injection. At 24 hr. nearly all cells of the liver showed much paler staining than normal, but by 48 hr. signs of restitution towards normal appeared in the liver lobules excepting the central necrotic areas. The liver cells which showed hydropic degeneration after one week still showed a reduced protein content by Dixon's method.

The mitochondria were examined 24, 48 and 72 hr. after the administration of tannic acid. These organelles disappeared from necrotic, or severely damaged cells, otherwise no conspicuous morphological changes were seen except for changes in the distribution of mitochondria within the cells. Whereas in normal liver cells the spherical mitochondria were fairly evenly distributed throughout the cytoplasm, after tannic acid intoxication the mitochondria were often situated around vacuoles and frequently at the periphery of the cells.

Succinic dehydrogenase activity decreased, mainly in the central parts of the lobules, as early as 30 min. after treatment. The loss of this enzymic activity progressed further so that by 6 hr. after the injection of tannic acid it almost completely disappeared in the central and mid areas of the lobules and was much diminished even at the periphery of the lobules (Fig. 3). Recovery of succinic dehydrogenase activity was noticeable around the central veins already at 12 hr., although at this time no formazan deposits could be seen in the mid areas of the lobules ; by 48 hr. the enzymic activity appeared almost normal throughout the liver lobules.

The histochemical test for lipase activity appeared in the control sections as irregular granules of various sizes scattered in various foci. In treated rats lipase activity at first (at 30 min.) increased and extended over entire liver lobules (Fig. 4). The substance representing the reaction product appeared in two forms : as coarse clumps and as fine granules. The early increase in lipase activity was followed by a precipitous fall, the reaction being completely missing 1 and 3 hr. after the injection of tannic acid. Weak lipase activity could be seen again at 6 hr. but at 48 hr. the activity observed exceeded that found in the controls. This increase was followed by another waning, particularly noticeable at 72 hr. and even 1 week after the injections the reaction products of lipase tests were scanty and found only in small foci.

Determination of glycogen content by biochemical procedures gave similar results to those found by histochemical techniques except that a fall in glycogen content could not be shown chemically 30 min. after the treatment. The minimum glycogen content was noticed at 6 hr., hereafter the content of this cell constituent fluctuated (Fig. 5) and by the end of 1 week the glycogen content of the liver was slightly higher than in the controls.

The paper chromatographic estimations of amino acids confirmed our previous observations (Horváth, Kovács and Korpássy, 1954). In a normal liver the following free amino acids could be demonstrated by our techniques : cystine,

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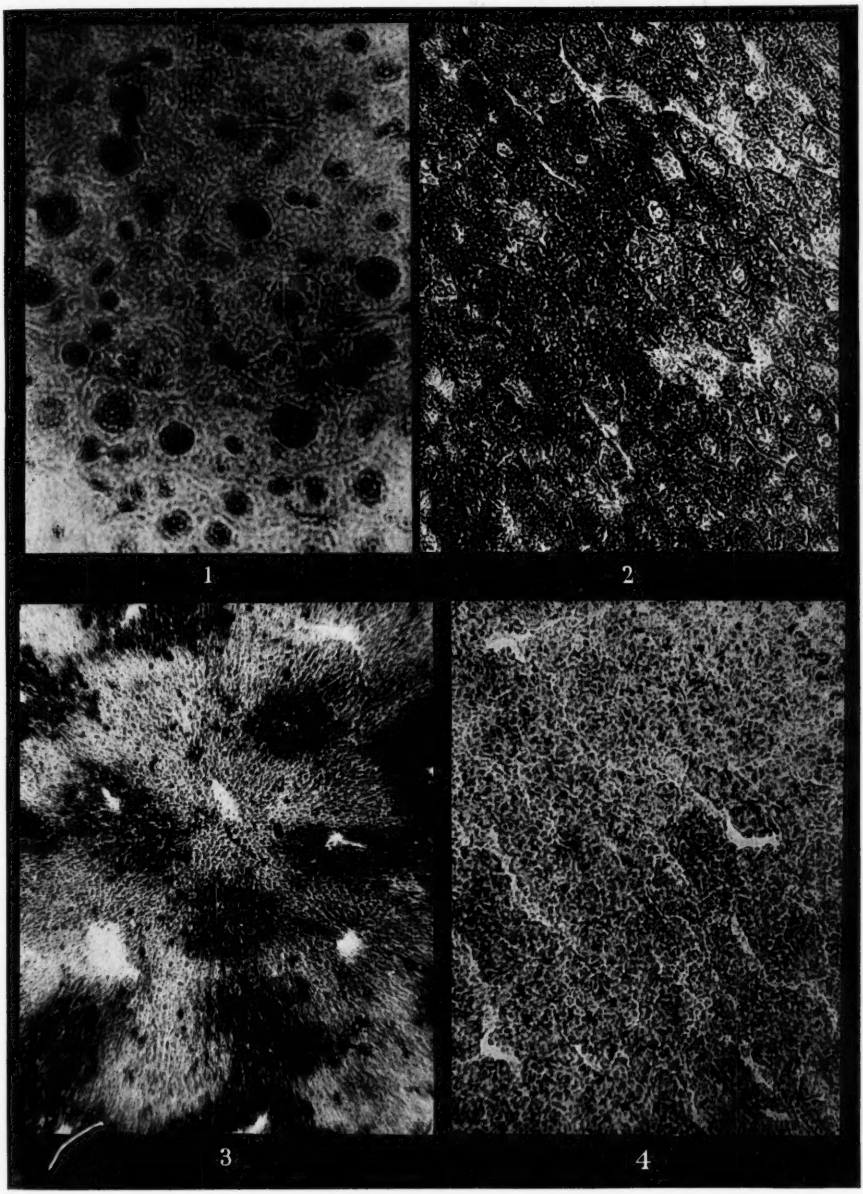
EXPLANATION OF PLATE

FIG. 1.—Liver section one week after the administration of tannic acid. Large cells with hyperchromatic oval nuclei are conspicuous. Feulgen's method. ×480.

FIG. 2.—Section of rat liver one hour after injection of tannic acid. There are several scattered pale staining cells in which the number of protein granules is much reduced. Tannin-azo method of Dixon. ×240.

FIG. 3.—Liver section 6 hr. after injection of tannic acid showing greatly reduced succinic dehydrogenase activity in central and mid areas of lobules. The darkly stained patches at the periphery of the lobules indicate surviving succinic dehydrogenase activity. ×40.

FIG. 4.—Liver section 30 min. after injection of tannic acid showing greatly increased lipase activity. The product of lipase activity is seen as fine and coarse dark granules of various sizes. ×88.



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very small amounts of cysteine, histidine, serine, glycine, glutamic acid and alanine. The amounts of cystine and cysteine increased and methionine appeared 24 hr. after tannic acid injection. At 48 hr. the spots of cystine, cysteine, glycine, glutamic acid, alanine and methionine became more intense, and

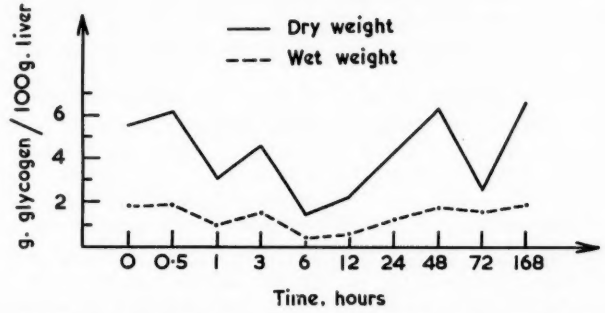

FIG. 5.—Changes in the glycogen content of liver after injection of tannic acid.

also norvaline, leucine, tyrosine and occasionally asparagine appeared. These changes were present to a lesser extent even at 72 hr., but one week after the treatment the free amino acid pattern was the same as in the controls.

Changes in the dry matter of the liver after injection of tannic acid are shown in Fig. 6 ; these ran parallel with the tissue lesions, the lowest value having been

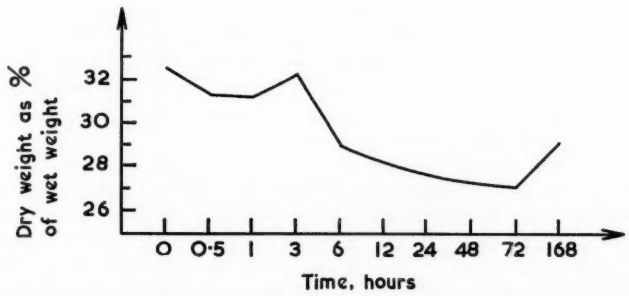

FIG. 6.—Changes in the dry matter of liver after injection of tannic acid.

found 48 and 72 hr. after the injections, but the normal level had not returned even 1 week after the treatment.

Paper electrophoretic examination of the serum proteins showed first a reduction in the proportion of albumin and a pronounced increase in the α_1 and α_2-globulins with no change in the β and γ-globulins (Table). Although the proportion of albumin returned to a nearly normal value one week after the injection of tannic acid, the α_1 and α_2 globulins remained elevated.

TABLE—*Relative Distribution of Serum Proteins in Rats After Injection of Tannic Acid*

Group	Number of rats	Albumin (%)	α_1-Globulin (%)	α_2-Globulin (%)	β-Globulin (%)	γ-Globulin (%)
Controls . .	5	57·24±1·15*	3·66±0·61	3·51±0·32	22·09±1·82	13·49±0.62
Tannic acid inj., 24 hr.	5	47·20±2·65	8·14±0·99	9·33±1·30	23·38±2·52	11·94±2·74
Tannic acid inj., 48 hr.	4	39·45±3·19	6·34±0·78	12·88±0·81	26·64±1·33	14·68±1·93
Tannic acid inj., 1 week	4	51·53±2·58	6·41±0·84	8·78±1·01	21·35±1·78	11·91±0·92

* Standard error of mean.

DISCUSSION

The results have shown that a single sublethal dose of tannic acid (250 mg./kg. body wt.) injected subcutaneously to rats gives rise to a marked and comparatively lasting lesion of the liver parenchyma which is associated with severe disturbances in various enzymic activities demonstrable by histochemical and biochemical procedures.

The rapid decrease in the glycogen content of liver tissue after the administration of hepatotoxic substances has been reported by several authors (Leduc and Wilson, 1958 ; Ruttner, Rondez and Gassmann, 1959). Apparently, this change is the most sensitive sign of hepatic lesion which is, in our opinion, only partly due to the disturbed metabolism and energy imbalance of liver cells, a prominent role being played by extrahepatic factors (stress) also. On the other hand, tannic acid induced liver damage is, strikingly, unattended by an increase of stainable fat, the phenomenon almost invariably associated with glycogen depletion caused by other agents injuring the liver (Myren, 1956 ; Eger, King and Schröder, 1958 ; Rüttner *et al.*, 1959). It may be inferred then that fatty degeneration is not an unconditional concomitant of toxic liver lesions associated with glycogen depletion. Evidently, its appearance depends on the action mechanism of the injuring substance.

This problem is closely related to the periodic changes observed by us in lipase activity. Gomori (1946) and other authors (Klein, Widmer and Grossmann, 1952) emphasize that a fall in the lipase activity of liver tissue is invariably followed by the increase of lipids, although Eger *et al.* (1958) found that this parallelism was not so close. Our observations have shown that a fall in lipase activity need not necessarily be followed by an increased neutral fat content. Fatty degeneration may be due to factors other than an inhibition of lipase activity.

The increase of free amino acids in the liver under our experimental conditions is not an unexpected phenomenon. It is a well-known fact that the lesion of liver cells leads to a disorder of nitrogen balance, to the reduction of deamination, wherefore amino acids appear in a higher number and quantity in the serum and urine (Dent, 1946 ; Walshe, 1953 ; Braun, Kisfaludy and Dubsky, 1954). An increased free amino acid content of the liver after treatment with tannic acid may result, of course, directly from destruction of protein in necrotic areas. Our histochemical examinations may be evaluated in the same sense, because the

sections stained by Dixon's method showed a reduced protein content in liver cells.

The mechanism of action of tannic acid cannot be defined yet. Himsworth (Himsworth and Glynn, 1942 ; Himsworth, 1950) believes that an impaired blood supply and tissue hypoxia could account for many toxic liver lesions. Without denying the significance of this factor, the changes observed in tannic acid poisoning cannot be explained by an impairement of tissue oxygenation. The nature and time of appearance of all the lesions suggests very strongly a direct effect of tannic acid on liver cells. Of late, an increasing number of authors claim that toxic cell lesions are, at least partly, sequels of the destruction of mitochondria (Christie and Judah, 1954 ; Leduc and Wilson, 1958). During our studies we have seen only an abnormal distribution of mitochondria within surviving cells without the appearance of morphological changes accessible to an examination with the light microscope. We cannot, however, say whether these structures, playing a very important role in cellular life, may not have suffered some functional damage ; changes in the succinic dehydrogenase activity suggest that they have suffered damage. Further biochemical investigations and studies with the electron microscope would greatly help in the elucidation of this problem.

The increase of α-globulins, which we noted after tannic acid poisoning, is generally held not to be a specific change, as it occurs with hepatic lesions and stress alike (Roberts and White, 1949 ; Roberts, 1953). This increase, the most characteristic change in the serum protein picture of animals poisoned with tannic acid, is in Scheurlen's (1955) view a simple concomitant phenomenon of disturbed cell metabolism. None of the observed changes gives a satisfactory explanation for this behaviour of α-globulins. In our opinion, it may be due to a summation of two effects : the nonspecific damage (stress) exerted by tannic acid, and its direct action on liver cells.

It appears from our experiments that the mechanism of action of tannic acid on the liver is different from that of the majority of known hepatotoxic substances, but it is not possible at present to define this mechanism.

SUMMARY

Albino rats were injected subcutaneously with a single non-lethal dose of tannic acid (250 mg./kg.) and their liver examined at various intervals after the injection.

Signs of hepatic lesions, such as swelling of cells, pyknosis, hydropic degeneration, acinocentral necrosis, were observed in addition to pronounced histochemical and biochemical changes.

Histochemical changes (decrease of liver glycogen, of succinic dehydrogenase and lipase activity, of protein content) appeared 30–60 min. after the administration of tannic acid, but after one week, the histochemical picture returned to nearly normal. Strikingly, no increase in the stainable fat content of the liver occurred at any time after tannic acid poisoning.

Tannic acid poisoning is associated also with an increase in the free amino acid content of the liver. In addition to the amino acids found in normal liver methionine, norvaline, leucine, tyrosine, and, less frequently, asparagine appeared after tannic acid injection.

Pronounced changes occurred in the distribution of serum proteins : decrease of albumin and great increase in α_1 and α_2-globulins, which lasted through the period of observation (1 week).

The observations were discussed in relation to the mechanism of action of various hepatotoxic agents.

Our thanks are due to Dr. G. Szepesi, Central Laboratory of the University Medical School, Szeged, for his help in the evaluation of the paper electrophoretic examinations.

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THE ROLE OF HISTAMINE IN EARLY BACTERIAL INFLAMMATION OF THE RAT PERITONEAL CAVITY

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Received for publication December 11, 1959

THE tissue mast-cell was established by Riley and West (1953) as an abundant reservoir of histamine, released it during the cell's degranulation or rupture. The peritoneum of the rat contains a large number of mast cells, highly susceptible to histamine-releasing agents given by intraperitoneal injection (Fawcett, 1954). The peritoneal cavity, being easily infected by pathogenic bacteria, is therefore eminently suitable for determining whether infective inflammation is accompanied by the release of histamine, and, if so, whether the histamine necessarily mediates the observed increase of vascular permeability. Some observations were also made on the release of 5-hydroxytryptamine (5-HT) during infective inflammation.

Our study was limited in two respects. First, it was confined to the first 4 hr. of infection; because, as judged by the reaction of guinea-pig skin (Burke and Miles, 1958) and rat skin (Miles and Wilhelm, unpublished) to infection, and of the rat pleural cavity (Spector, 1956) to turpentine inflammation, the major increases in vascular permeability occur during this period. Second, the inflammation was induced by minimal effective doses of bacteria, to avoid gross tissue damage, in which the release of histamine that was in essence irrelevant to natural inflammation might complicate the interpretation of results.

MATERIALS AND METHODS

Bacteria and toxin.—The bacteria used were: *Staphylococcus aureus* NCTC 491, *Corynebacterium ovis* 1/50 (from Professor H. R. Carne) and *Pseudomonas pyocyanea* (Scudder). Staphylococcal toxin was prepared by growing *Staph. aureus* strain 5AS on sloppy nutrient agar for 24 hr. at 37° in the presence of 20 per cent CO_2. The toxin was partially purified by a procedure similar to that of Wittler and Pillemer (1948). Fifteen per cent methanol was added to the crude toxin at − 5°, previously brought to pH 4·3 by the addition of glacial acetic acid; the resulting precipitate was removed by centrifugation and resuspended in phosphate buffer pH 7·5 to one tenth of the original volume. Staphylococcal antitoxin 362A is a refined globulin preparation obtained from the Wellcome Laboratories.

Rats.—The animals, bred from the albino strain of the Chester Beatty Institute, weighed about 200 g.

Bacterial inoculum.—The organisms in 1 ml. of 18 hr. nutrient broth cultures were washed × 3 by centrifugation in de Jalon's isotonic salt solution and suspended in 15 ml. of the same solution. Before injection the inoculum was warmed to 37°. The control inoculum contained thrice-washed organisms killed by heating for 30 min. at 60°.

Procedure.—The inoculum was injected intraperitoneally through the left lower quadrant of the abdominal wall and the animals killed by stunning after periods of up to 5 hr. Immediately after death the peritoneal cavity was opened and drained of fluid; the cavity was then washed out with 10 ml. of de Jalon's salt solution, and the washings added to the fluid already

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removed. The total volume was determined and the fluid centrifuged to remove cells, fibrin and most of the bacteria.

Protein.—Protein was estimated by U.V. absorption in a Unicam S.P. 500 spectrophotometer. The maximum absorption by the peritoneal fluids and by rat serum occurred at a wavelength of 280 mμ. The optical density ($\text{Log}I_0/I$) of rat serum diluted 1/100 was 0·67, and the protein nitrogen content by micro-Kjeldahl 0·11 mg./ml. As a routine the centrifuged fluid was diluted 1/10, placed in a 1 cm. quartz cell and optical density (OD) at 280 mμ determined. For the purpose of this investigation the absolute figures for protein were not required, only the relative amounts in test and control animals. For this reason only the optical densities (OD) of fluids diluted 1/10 are cited. That absorption of light at 280 mμ was due almost entirely to protein in the peritoneal fluid was evident from its virtual disappearance from the fluid after precipitation of protein by trichloracetic acid. That absorption was proportional to protein concentration was confirmed by micro-Kjeldahl estimates. Total protein in peritoneal fluid is expressed as the product of its volume and the optical density of 1/10 (V × OD). As a rough guide, a V × OD value of 1·0 in the tables corresponds to 10–11 mg. protein in the combined peritoneal fluid and washings; the correspondence to other values is directly proportional.

Histamine was estimated using guinea-pig ileum suspended in atropinized Mg.-free Tyrode solution in an 18 ml. organ bath. Estimation of 5-HT was carried out on isolated rat colon suspended in de Jalon's solution.

Increased capillary permeability induced in the skin of rats by histamine was measured as described by Sparrow and Wilhelm (1957).

Drugs.—Histamine was used as the acid phosphate and weights are cited as base. Triprolidine (" Actidil ") was supplied by Burroughs Wellcome.

Staining of Mast Cells.—Mesenteric peritoneum was fixed in methanol, and stained with 1 per cent toluidine blue.

RESULTS

Peritonitis Caused by Bacterial Infection

Two of the organisms, *Ps. pyocyanea* and *C. ovis*, consistently induced typical inflammatory changes in the peritoneum within a few hours of inoculation. *Staph. aureus* had a slighter effect. In the dose used, *Ps. pyocyanea* and *C. ovis* killed the animals within 24 hr. but the Staphylococcal infection was not lethal.

During the first 4–5 hr. rats receiving live bacteria differed little in external appearance from those receiving heat-killed organisms. In some experiments with *Ps. pyocyanea*, the rats were quieter than the controls and sat huddled up with staring coat. Animals killed during this period appeared to be normal; in some, the peritoneal blood vessels were more injected than in others but this was an inconstant finding, and was not consistently associated with increased protein in the peritoneal exudate (see below). In both the experimental and the control series the number of polymorphonuclear leucocytes in the peritoneal fluid was increased to about the same degree after 2 hr. and it was assumed that this cellular response had been provoked by the large volume of injection fluid and not by the organisms suspended in it. During a 4 hr. period, staphylococci, but not the pseudomonads, were taken up by phagocytes.

Protein in the peritoneal fluid

The most striking and constant finding in animals injected with live bacteria was the higher protein content of the peritoneal fluid, compared with controls receiving dead organisms. The difference in peritoneal protein between animals receiving live and dead organisms first becomes substantial 3–4 hr. after inoculation. Table I exemplifies results in staphylococcal infection. In most experiments, therefore, the animals were killed after this interval (Table II).

TABLE I.—*The Protein and Histamine Content of the Peritoneal Fluid of Rats at Varying Intervals after Peritoneal Infection with* Staph. aureus, *and the Residual Peritoneal Histamine Liberated by the Drug* 48/80

Expt.	Time killed (hr.)	Volume (V*) (ml.)	Protein		Histamine	
			OD*	Total OD × V	In fluid (μg.)	Released by 48/80 (μg.)
1. Infected	0·5	22·5	0·13	2·92	1·35	15
Control	0·5	21·0	0·13	2·73	1·26	20
2. Infected	3·0	16·0	0·40	6·40	1·60	15
Control	3·0	1·6	0·31	4·96	0·90	10
3. Infected	4·0	14	0·41	5·74	1·40	15
Control	4·0	13	0·37	4·81	0·80	10

* See p. 306 for the explanations of these values in Table I–VI.

TABLE II.—*The Protein and Histamine Content of Peritoneal Fluid of Animals Killed* 4 *hr. after Peritoneal Inoculation with Different Bacteria*

Pathogen	Number of animals	Protein (OD × V)		Histamine (μg.)	
		Average	Range	Average	Range
Staph. aureus	12	6·68	4·5–9·8	1·18	0·2–3·2
Staph. aureus (heat-killed)	9	4·55	3·1–6·6	0·78	0·4–0·9
C. ovis	10	9·20	8·2–14·8	1·19	0·6–3·2
C. ovis (heat-killed)	5	4·44	4·0–5·1	1·47	0·3–3·0
Ps. pyocyanea	11	9·79	5·9–16·5	1·24	0·6–2·0
Ps. pyocyanea (heat-killed)	7	4·20	3·8–5·8	1·27	0·3–2·2

Both *Ps. pyocyanea* and *C. ovis* induce the exudation of a consistently greater amount of protein than do staphylococci. In each test with staphylococci the protein induced by live organisms was always more than in the controls, but between different experiments the highest control value was sometimes higher than the lowest in infected animals. This was never so with the inflammation induced by *Ps. pyocyanea* or *C. ovis*.

Histamine in the peritoneal fluid

The total histamine in the peritoneal fluid of animals injected with live or killed organisms, was at all times usually less than 2 mg. In 17 experiments in which the rats were given 15 ml. de Jalon's solution alone, the amount was also less than 2 mg. Larger amounts occasionally appeared in both infected and control animals, and were possibly due to trauma inflicted during the experimental procedures. It is clear (Tables I and II) that bacterial inflammation, with high protein exudation presumably due to increased capillary permeability, is not associated with the presence of increased amount of histamine at the site of inflammation.

Histamine remaining in the peritoneal wall

The histamine content in the peritoneal fluid was remarkably constant during the first 4 hr. of infection and did not exceed that in control animals. It was

possible that throughout this period histamine was constantly liberated and simultaneously destroyed, or drained away, thus masking a greater liberation of histamine in the infected animals. To check this point, the liberable histamine remaining in the peritoneal tissue was estimated. Fifteen ml. of de Jalon's solution containing the histamine liberator 48/80 (15 μg./ml.) was injected into the peritoneal cavities of animals of both groups after killing; after 20 min. the fluid was removed and its histamine content measured. The injection of 15 ml. de Jalon's solution, with or without added bacteria, liberated 0·8–1·6 μg. histamine (Tables I and IV); and some 20 μg. were liberated by subsequent

TABLE III.—*The Protein Content of Peritoneal Fluid 30 min. after the Intraperitoneal Injection of 15 ml. Fluid Containing Varying amounts of Histamine. One Rat per Dose of Histamine*

Histamine injected (μg.)	Peritoneal fluid			
	Volume (V) (ml.)	Protein (OD)	Total (OD × V)	Histamine content (μg.)
0	22	0·15	3·3	0·4
1	19	0·17	3·23	1·0
5	16	0·15	2·4	3·2
10	22	0·14	3·08	4·4
25	20	0·16	3·2	4·0

TABLE IV.—*The Protein and Histamine Content of Peritoneal Fluid Half and One Hour After the Intraperitoneal Injection of 15 ml. Fluid Containing 225 μg. of the Histamine Liberator, 48/80*

Treatment	Duration of treatment (hr.)	Peritoneal fluid			
		Volume (V) (ml.)	Protein (OD)	Total (OD × V)	Histamine (μg.)
48/80	0·5	22	0·21	4·62	13·2
	0·5	23	0·13	2·99	11·5
	1·0	15	0·14	2·10	4·5
	1·0	23	0·12	2·76	9·2
Controls	0·5	21	0·18	3·78	1·36
	1·0	17·5	0·27	4·72	0·97

treatment with 48/80, irrespective of the nature of the previous injection—live or killed staphylococci (Table I), live *Ps. pyocyanea* or *C. ovis*. Some 20 μg. were also liberated in previously untreated animals receiving 48/80 *in vivo* (Table IV). It is therefore unlikely that amounts larger than those detected in the peritoneal fluid were liberated during the infections. All the experimental procedures, control or other, released 7–9 per cent of the total histamine liberable by test injection and 48/80 combined.

The possibility was also examined that the peritoneal fluid of infected or control animals contained histaminopectic substances (Parrot and Laborde, 1956) or substances, such as histaminases, capable of inactivating histamine. Histamine 1 μg./ml. in de Jalon's solution, pH 7·2, was incubated *in vitro* at 37° with an equal volume of a number of peritoneal exudates from infected and control animals for periods up to 2 hr.; the amount of histamine was unaltered.

Peritoneal fluid 5-HT

Combined exudate and washings, harvested at 3–4 hr. from 30 of the infected and 17 of the control animals, consistently contained less 5-HT than we could detect *in vitro* with preparations of rat colon; *i.e.* 0·02–0·05 μg./ml. In a few early tests with exudate alone, undiluted by subsequent peritoneal washings, amounts up to 0·06 μg./ml. were detected. Assuming an average volume of 20 ml. for combined exudate and washings, we may reasonably conclude that less than $20 \times 0{\cdot}05 = 1$ μg. 5-HT was released into the peritoneal cavity under the stimulus of either de Jalon's solution or the infecting microbes.

Peritonitis Caused by Staphylococcal Toxin

The course of peritonitis induced by a bacterial toxin, the α-toxin of *Staph. aureus* (Table V), was compared with that induced by bacteria. The toxin preparation was dissolved in 15 ml. of de Jalon's solution and injected intraperitoneally; the animals were killed 4 hr. later. Two animals received toxin

TABLE V.—*The Protein and Histamine Content of Peritoneal Fluid of Animals 3·5 hr. after the Intraperitoneal Injection of* Staph. aureus *α-Toxin*

Animal No.	Toxin (ml.)	Antitoxin (I.U.)	Peritoneal fluid			
			Volume (V)	Protein (OD)	Total (OD × V)	Histamine (μg.)
1	0·125	0	14·0	0·35	4·9	2·8
2	0·125	0	18·5	0·38	7·03	0·92
3	0·25	0	22·0	0·56	12·32	1·01
4	0·25	0	22·0	0·57	12·54	0·88
5	0·25	10	13·5	0·27	3·64	1·35
6	0·25	10	13·5	0·34	4·59	0·81

neutralized by 10 International Units (I.U.) of antitoxin (as indicated by inhibition of haemolysis of rabbit R.B.C.). The largest amount of toxin caused pronounced congestion of the peritoneum within 4 hr. and death of the animal within 24 hr. Despite severe congestion of the mesenteric and omental blood vessels and the high protein content of the peritoneal fluid, the mast cells were histologically normal and the fluid contained little histamine. The prevention of the inflammation by antitoxin suggests that the changes were attributable to the α-toxin.

From these few experiments it appears that peritonitis due to staphylococcal toxin resembles that due to live bacteria in that there is a substantial exudation of protein, associated with the liberation into the peritoneal fluid of no more histamine than occurs after the injection of de Jalon's solution.

Relation of Intraperitoneal Histamine to Protein Exudate

In the skin of rats large amounts of histamine must be injected to demonstrate increased capillary permeability. From the results of Sparrow and Wilhelm (1957) it appears that about 30 μg. are required for the vessels in 1 sq. cm. of skin. The 2 μg. amounts found in the peritoneal exudates were small by comparison. It is however possible that increased permeability to blood

proteins is more readily induced by a given dose of histamine in the more exposed and more extensive vessels in the walls of the peritoneal cavity. The possibility was tested in normal animals, first with known amounts of histamine and second with the histamine liberator 48/80.

Effect of injected histamine.—The injection of histamine in doses ranging from 1 to 25 μg., an amount greatly exceeding that found in the experimental infections, did not increase the exudation of protein (Table III).

Effect of a histamine-liberator.—Rats were given 15 ml. of de Jalon's solution containing 48/80 (15 μg./ml.) intraperitoneally and killed after 30–60 min. Although after this period the histamine content of the peritoneal fluid was much greater than in controls receiving de Jalon's solution alone, the protein content was similar in both groups. Clearly the liberation of endogenous histamine cannot account for leakage of protein to the extent found in bacterial peritonitis.

Effect on inflammation of an antihistamine drug

Although the experimental infections studied do not appear to liberate histamine into the peritoneum, histamine, in the presence of live organisms, may nonetheless play an essential role in the inflammatory events. We were, however, unable to detect any change in the exudation of protein when infection was induced in rats treated with the powerful antihistamine triprolidine (Mason and Wilhelm, unpublished). In an intramuscular dose of 2·5 mg./kg., triprolidine entirely suppressed the increased capillary permeability induced in the skin of rats by 5·4 and 16·2 μg. histamine, given intracutaneously 2 hr. later in a volume of 0·1 ml. For the infection experiments, triprolidine was given intramuscularly, in 2 doses of 2·5 mg./ml. Two groups of animals received live organisms intraperitoneally in 15 ml. of de Jalon's solution. A third received 15 ml. of solution without organisms. This control group and one of the infected groups received

TABLE VI.—*The Effect of Intramuscular Triprolidine 5 mg./kg. on the Protein Exudation Present 4 hr. after the Intraperitoneal Injection of Living* C. ovis.

Treatment	Peritoneal fluid		
	Volume recovered (V)	Protein (OD)	Total (OD × V)
C. ovis and triprolidine	18	0·60	10·8
	17·5	0·50	8·7
	16	0·58	9·4
	16	0·47	7·5
C. ovis	18·5	0·80	14·8
	17·5	0·59	10·3
	16	0·54	8·7
	16	0·58	9·4
Triprolidine	15	0·39	5·9
	14·5	0·42	6·5

the triprolidine 2 hr. before and again 2 hr. after the injection of bacteria. Table VI shows the results with a *C. ovis* infection 3 hr. old. Similar results were obtained with *Ps. pyocyanea*. It is evident that the antihistamine did not alter the inflammatory response, as indicated by exuded protein.

Mast Cells and Infective Inflammation

Immediately after the animals were killed and the peritoneal fluid drained off, portions of clear mesentery were spread over small coverslips excised and immediately fixed and stained. No difference in morphology or staining reactions were detected between the mast cells from infected animals and from animals which had received killed organisms, staphylococcal toxin, or de Jalon's solution alone. In all groups of animals the cells were unruptured and had retained their normal granular appearance.

DISCUSSION

There are several indications that histamine and 5-HT have only a minor and perhaps inessential role in the early vascular reactions to injury. Thus, in the rat turpentine releases histamine and 5-HT soon after its introduction into the pleural cavity: but both disappear from the pleural fluid after 30–60 min., whereas the rate of exudation of proteins reaches its maximum several hours later; and the suppression of the 2 substances by appropriate specific inhibitors does not alter the main sequence of inflammatory events (Spector and Willoughby, 1957, 1958, 1959). When the skin of the rabbit, rat or guinea-pig is mildly injured by heat or ultraviolet light, there follows a biphasic increase in vascular permeability; a short-lived immediate response totally inhibitable by antihistamine drugs, and a major delayed response quite unaffected by them (Wilhelm and Mason, 1958; Wilhelm, unpublished). Progressive injury by living pathogenic bacteria induces a similar biphasic response in the skin of the guinea-pig (Burke and Miles, 1958); though the immediate response which by analogy might be due to histamine, is largely eliminated by thorough washing of the bacterial inoculum. As far as we examined them, the time-relations of the permeability response to infection in the rat peritoneal cavity were similar, with a major exudation of plasma protein at the 3rd and 4th hr., and no evidence that washed bacteria induced an early histamine-like effect. In 1 non-lethal, and 2 ultimately lethal infections, the exudation of protein and cells bore no relation to the release of histamine or 5-HT. Indeed, the negligible amount of 5-HT and the small amount of histamine found were released as effectively by the relatively large volume of the isotonic salt solution used, as by salt solution containing well washed living bacteria or a lethal dose of staphylococcal toxin; in no case was the amount of histamine released more than 10 per cent of the total histamine released by a powerful histamine liberator; and, in the doses employed, none of the injurious agents affected the integrity either of the peritoneal mast cells or their granules. Moreover, the systemic administration of an antihistamine drug failed to affect the subsequent course of bacterial inflammation as indicated by protein exudation.

Certain inflammatory agents—like egg white (*e.g.* Schachter and Talesnik, 1952)—release histamine. One bacterial toxin, the α-toxin of *Clostridium welchii*, is a potent destroyer of mast cells in the rat peritoneum (unpublished). This toxin is a lecithinase C, and it is noteworthy that according to Högberg and Uvnäs (1957), endogenous lecithinase A is a key enzyme in mast cell degranulation and rupture. But the substantial release of histamine is peculiar to relatively few inflammatory agents, and as evidence for the general significance of histamine in inflammation is almost wholly question-begging.

In our results there is therefore no ground for any generalization about the essential role of histamine in the first few hours of natural inflammation, or for assigning to histamine-release by the mast cell any general significance in responses to injury. Considered as a purposive response, the release of histamine and 5-HT appears in many cases to be epiphenomenal.

SUMMARY

Ultimately lethal infections were induced in the rat by intraperitoneal *Ps. pyocyanea* and *C. ovis*, a non-lethal infection by *Staph. aureus* and a lethal intoxication by *Staph. aureus* α-toxin. All 4 agents, given in 15 ml. de Jalon's solution, induced typical inflammation, with substantial exudation of protein and cells, during the 3rd and 4th hours. During the first 4 hr. only about 10 per cent of the liberable histamine appeared in the peritoneal fluid, apparently released by the stimulus of the large volume of fluid injected. No more was released by the microbial or toxic stimuli.

Only negligible amounts of 5-hydroxytryptamine were found in the peritoneal fluid. The mast cells of the mesentery and omentum were morphologically unchanged during the infection.

The inessential role of histamine in the early reactions to acute infection thus indicated was confirmed by the failure of the antihistaminic drug triprolidine to modify the inflammatory process.

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GROWTH AND TOXIN PRODUCTION OF STAPHYLOCOCCI IN CELLOPHANE SACS *IN VIVO*

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Received for publication December 14, 1959

IT has been repeatedly pointed out (*e.g.* Dubos, 1954) that 2 distinct conditions determine the growth of bacteria *in vivo*. Firstly, the animal tissues must provide a suitable medium and conditions for growth, and secondly the bacterium must be capable of resisting the antibacterial factors in the host. These 2 conditions cannot be well differentiated in any tests in which the organism is introduced directly into the tissues. However, an investigation into the first condition, the ability of the tissues to support growth, is made more possible by introducing the organisms into the animal inside a cellophane sac. All known growth factors are small enough to pass through the wall of the sac (M.W. less than about 20,000), whereas cells, antibacterial factors associated with serum proteins (Ekstedt, 1956) and many, but by no means all, other antibacterial factors would be excluded. In addition, extracellular toxins, enzymes and other proteins produced by the organism would be retained inside the sac, so that the production of these substances *in vivo* could be investigated.

It has not been possible to find in the literature any reference to the use of cellophane sacs *in vivo*. Collodion sacs have, however, been used extensively (see Gates, 1921, 1925 ; Ficker, 1927–29 ; Harris, 1939 ; Harris and Miller, 1941) particularly to exclude phagocytes (Harris and Miller, 1941) and in the early attempts to cultivate viruses and rickettsiae in the absence of cells (Olitzky and McCartney, 1923). The porosity of the sacs used is seldom stated, but there is no evidence that sacs of sufficiently small pore size to hold back proteins have ever been used. On the contrary the passage of diphtheria toxin (Miller, 1911), antigens (Ficker, 1927–29) and even the slow growth of bacteria through the wall of the sac (Harris and Miller, 1941), have been described.

In the present work, staphylococci have been grown in cellophane sacs in the peritoneal cavity of rabbits, guinea-pigs, mice and rats, their growth curves plotted, and their production of α haemolysin, P.V. leucocidin and coagulase estimated.

A preliminary report of this work was communicated to the 7th International Congress of Microbiology (Glencross and Gladstone, 1958).

METHODS

Strains of Staphylococci.—Most of the work was carried out with 5 strains of coagulase positive staphylococci : V8, isolated by the late Dr. F. P. O. Valentine from a patient with furunculosis and used in earlier work on leucocidin (Gladstone and van Heyningen, 1957). This strain was kindly typed for us by Mr. V. G. Alder and gives the pattern 52/52A/79/80/

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7/42E/54 + ; P 80, a Phage type 80 strain ; Newman, used by Duthie and Lorenz (1952) in studies on coagulase. This strain tends to throw off proteoloytic variants producing little coagulase. A coagulase positive non-proteolytic strain was accordingly selected by plating on fibrinogen-agar (Klemperer and Haughton, 1957) ; Oxford, the well known strain used in the assay of penicillin ; Heath, a strain freshly isolated from a patient with osteomyelitis by Dr. A. G. Towers. The P80 and Newman strains were kindly supplied by the late Dr. E. S. Duthie. In addition, 12 strains from patients with osteomyelitis obtained from Dr. A. G. Towers of the Royal National Orthopaedic Hospital, 5 freshly isolated coagulase positive strains which failed to produce α haemolysin and 7 strains of coagulase negative staphylococci were used. All strains were preserved in the dried state.

Animals.—These were from the laboratory stock. All animals were well grown, mice greater than 16 g. being selected.

Cultural Methods.—Inoculum. A master culture on a tryptic-heart agar slope from the dried organisms, renewed at intervals of 6–8 weeks, was used to inoculate a tryptic-heart agar slope for each experiment. A suspension in saline was made from the overnight growth of this culture, and diluted to contain a known number of organisms, estimated either by opacity or viable counts by the methods stated below.

Preparation of Sacs

Well washed Visking cellophane dialysis tubing, 1 cm. wide (folded flat), was used throughout the work.

Without Facilities for Sampling.—In the initial experiments, sacs were made by tying a knot in one end of tubing of the required length and tested for leaks by water pressure. A known volume of saline was then added. For sterilization, the sac was placed in a test tube to which saline was added until the level outside was the same as that of the saline inside the sac. The test tube was plugged with wool and autoclaved at 15 lb. for 20 min. The sac was then removed with aseptic precautions and inoculated with a known number of organisms using a Pasteur pipette. The free end of the sac was then knotted on itself with sterile forceps, the knot being immersed in boiling water for 2 min. to destroy any organisms likely to have contaminated the outside at the time of tying off, and the sac replaced in the glass tube ready for insertion into the animal.

Owing to difficulty in preventing contamination of the outside of the sac, which immersion of the knot in boiling water did not entirely prevent, an improved procedure was adopted for later experiments. A sac was prepared and tested for leaks as before but, before autoclaving, the free end was closed with a rubber bung just fitting the cellophane tube and held in place with a tight rubber band. A No. 20 hypodermic needle was inserted through the rubber bung into the sac. The sac was then autoclaved empty, or with the requisite amount of saline, and inoculated with a syringe through the needle in the bung. The needle was then withdrawn, a flame being applied to the surface of the bung and the rubber slightly burnt to destroy any organisms deposited on it at the time of withdrawal. Sterility tests made by placing such sacs in nutrient broth were generally successful.

With Facilities for Sampling in situ.—Where it was necessary to obtain repeated samples of the contents of the sac during an experiment, as for instance in plotting growth curves, the apparatus was modified as in Fig. 1. Nylon tubing gauge 0 (bore about 0·2 mm.; from

FIG. 1.—Cellophane sac fitted with nylon tube for inoculation and sampling. For description see text.

Portland Plastics Ltd., Hythe, Kent) (A) was passed through the rubber bung (B) by threading it through the eye of a sewing needle and lubricating the needle and tube well with glycerol. The bung was placed in the mouth of the sac (C) and held in place by a tight rubber band (D) the end of the tube reaching to the bottom of the sac. A No. 20 hypodermic needle (E) was inserted into the lumen of the free end of the nylon tube. This was of a sufficiently small bore to grip the needle firmly and to resist considerable water pressure from a syringe

used for testing for leaks. The sac with the nylon tube and needle attached was placed inside a glass test-tube and autoclaved empty. The sac was filled and inoculated after it had been inserted into the animal. The needle was then closed with a stopper (F) made from a Record-Luer needle adaptor blocked with solder. The nylon tube had negligible capacity, so that inoculation and sampling could be made through a relatively long tube without having to allow for the dead space.

Insertion of Sacs into the Peritoneal Cavity of Laboratory Animals.—The animal was anaesthetized and a median incision made through the abdominal wall. The sac was inserted and the wound closed with silk sutures. If a nylon sampling tube was attached to the sac, this was led out of the wound and secured to the back (rabbits and guinea-pigs) or tail (mouse). In rabbits as many as 8 sacs, 4 cm. long, with sampling tubes attached, could be placed in the same animal, each sac containing 3 ml. of fluid. In such cases, the nylon tubes, instead of being led out through the operation wound, were passed separately through the abdominal wall well up on the flanks of the animal. This was done by removing the hypodermic needles at the end of the nylon tubes, threading the tubes through the eye of a sterile suture needle and passing the suture needle through the abdominal wall from within outwards. The hypodermic needles with their stoppers were then replaced in the free ends of the nylon tubes, the tubes labelled with adhesive tape and a collodion dressing placed over the operation wound. A convenient method of securing the tubes on the back of the animal is to attach the needles to a needle holder from a box of No. 20 needles as shown in Fig. 2. As an additional precaution, to prevent the animal from reaching the tubes with its mouth, a cardboard collar was fitted. With mice the needle was secured to the tail with adhesive tape, no slack being allowed. Aseptic precautions are particularly important since the sac, as a foreign body, has an enhancing effect on infection (Elek, 1956).

Inoculation.—Sacs fitted with nylon sampling tubes were usually inoculated on the day following the operation. A known number of organisms (usually 10^5/ml.) was inoculated through the nylon tube with a syringe. As the fluid was often under pressure, it was necessary to clip the tube with artery forceps when the syringe was removed. The plug could then be re-fitted and the clip removed. Closed sacs were of course inoculated before insertion. The maximum volumes used were 2 ml. in mice, 10 ml. in rabbits and 5 ml. in guinea-pigs and rats.

Estimation of Growth

When growth curves were plotted, or when the opacity of the culture was below that able to be recorded by opacity measurements, viable counts were carried out by the method of Miles and Misra (1938). Estimations of heavier growth were made on a Spekker absorptiometer calibrated in terms of dry weight of organisms. With volumes too small to be estimated by Spekker absorptiometer, direct counts in a Helber counting chamber were made.

Assay of α Haemolysin

α haemolysin was assayed by an antitoxin combining method with standard α antitoxin (20 u./ml.; from the Biological Standards Laboratories, National Institute for Medical Research, Mill Hill).

Since samples were often small in volume (0·1 ml.), a semi-micro technique was developed. The usual procedure, in which dilutions of an unknown amount of toxin are added to a fixed dose of standard antitoxin, was reversed, the standard antitoxin being diluted in 50 per cent steps in 0·5 per cent gelatin saline (usually from 1·0–0·01 u./ml.). A fixed arbitrary volume of each dilution (about 0·03 ml.) was pipetted onto glazed waxed tiles using a capillary tube attached to an automatic spring-loaded delivery micro-pipette. The same volume of the test sample, usually diluted 1/10 without centrifuging or filtering, was then added to each dilution of antitoxin. After thorough mixing and after allowing to stand for 30 min., the same volume of 3 per cent washed rabbit red cells was added, and the mixture run up by capillary attraction into fine bore capillary tubes (vaccine lymph tubes as used by van Heyningen and Gladstone, 1953). The tubes were sealed in a flame, placed in a suitable rack and incubated in a water bath for 1 hr. at 37°.

Assay of P.V. Leucocidin

A semi micro-procedure modified from that of Towers and Gladstone (1958) was used. As with the haemolysin assay, the normal procedure was reversed, falling dilutions of the

standard antitoxin being added to a constant amount of the toxin to be assayed. The standard was antitoxin CPP 76/63 from the Wellcome Research Laboratories, Beckenham, arbitrarily fixed at 240 units antileucocidin/ml. (Gladstone and van Heyningen, 1957 ; Woodin, 1959). This was diluted in 50 per cent steps (usually from 1 to 0·01 u./ml.) and small volumes (approximately 0·02 ml.) of these dilutions added to the same volume of the sample (usually diluted 1/10 ; not centrifuged or filtered) on microscope slides. After mixing with a platinum loop and allowing to stand, cover glasses containing adherent human leucocytes (Gladstone and van Heyningen, 1957) were placed on each mixture, sealed by paraffin wax, incubated at 37° for 30 min. and examined under light phase contrast. The end point was the first preparation in the series to show the changes in the leucocytes described by Gladstone and van Heyningen.

Assay of "Fast" and "Slow" Components of Leucocidin.—During the course of the present work, Woodin (1959) showed that the activity of P.V. leucocidin was due to 2 synergistic proteins which he obtained in a crystalline state, electrophoretically and antigenically homogeneous. He named them " Fast" (F) and " Slow" (S) according to the rate at which they migrate on a column of carboxymethyl cellulose.

In this work, in all estimations of leucocidin made subsequent to this discovery, both components were assayed separately. The procedure was as described above, except that 2 series of assays were carried out, one in the presence of excess " F " (that is excess over and above the amount neutralised by the amount of F antibody in a particular dilution of the standard antiserum) for the assay of " S " and vice versa. For this purpose purified preparation of " F " and " S " components were obtained from Dr. Woodin. The procedure is outlined in Table I.

TABLE I.—*Assay of " Fast " (F) and " Slow " (S) Components of P.V. Leucocidin*

Reagents : Antileucocidin CPP 76/63; (240 u anti F/ml.); (240 u anti S/ml.) Arbitrarily fixed
F (free from S) 10 L +/ml. with excess S
S (free from F) 10 L +/ml. with excess F

Assay of S

Anti-leucocidin u/ml. :	1·0	0·66	0·44	0·3	0·2	0·1	0·087	0·058	0·039	0·026	0·017	0·011
* F L +/ml. :	10	6·6	4·4	3	2	1	0·87	0·58	0·39	0·26	0·17	0·11

Sample for assay : Constant dilution (usually 1/10).

Equal volumes (about 0·02 ml.) of each dilution of antileucocidin mixed with the same volume of the appropriate dilution of F on clean glass slides, and the same volume of the sample dilution added. Preparations of human leucocytes on cover glasses placed over each mixture and the changes in the cells observed microscopically (Gladstone and van Heyningen, 1957).

Assay of F : as above, but constant excess of S used in place of F.

* Constant excess : L+ units F/units antitoxin = 10/1. This ratio, however, is not critical.

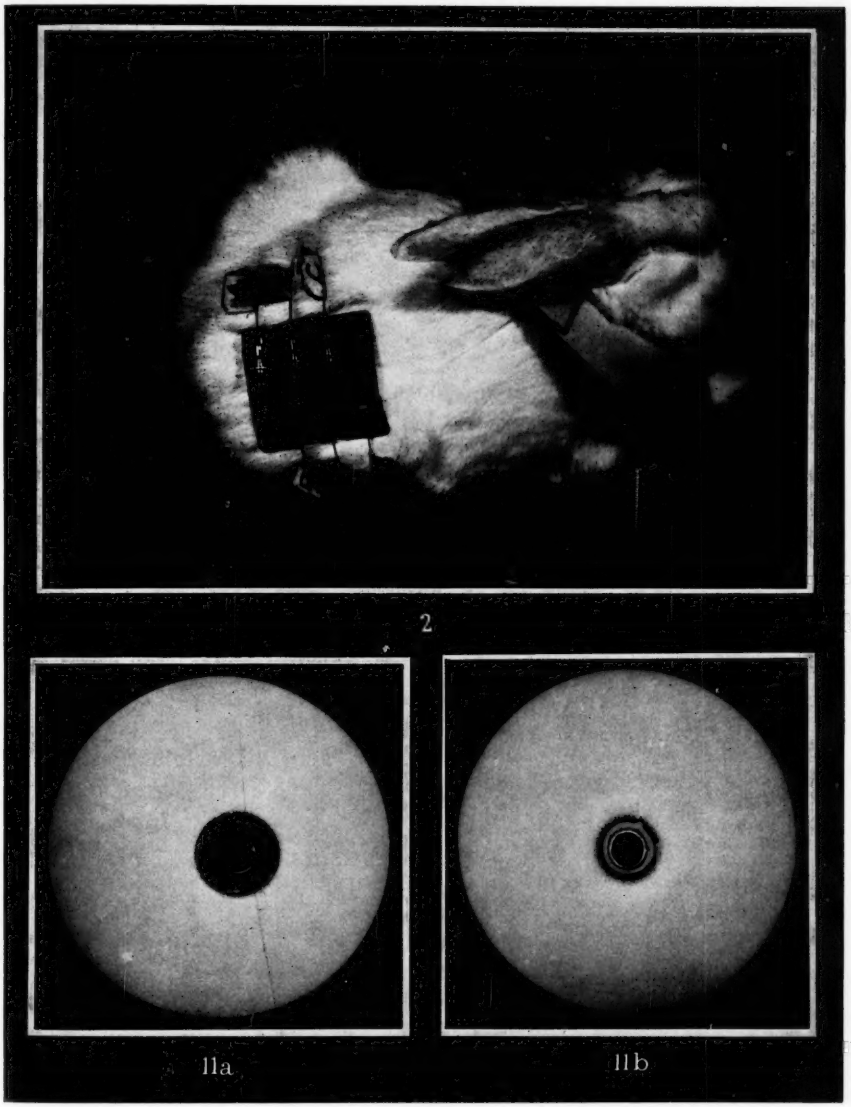
Assay of Coagulase

Coagulase was estimated by a modification of the method of Duthie (1954). Human plasma was diluted 1/5 with saline containing 0·3 per cent sodium citrate and 0·75 per cent Filter-Cel. Volumes of 0·02 ml. were distributed in capillary tubes using an automatic spring loaded delivery syringe attached to a micro-Pasteur pipette, and kept in the water bath at

EXPLANATION OF PLATE.

FIG. 2.—The arrangement of sampling tubes from 6 sacs in the peritoneal cavity of a rabbit. The cardboard collar prevents access to the tubes and operation wound with the animal's mouth.

FIG. 11.—Test for lysozyme activity of pleural exudate and pleural exudate diffusate. Medium : Difco heart infusion agar bulk-seeded with *M. lysodeikticus*. The wells in the centre of the plates contained : A, pleural exudate and B, the contents of a sac from a culture of strain P80 in a diffusate of pleural exudate before visible growth was observed. The ring of growth round the well in B is that of P80. Tests showed that P80 had no inhibitory effect on the growth of *M. lysodeikticus*.



2

11a

11b

Gladstone and Glencross.

37°. Equal volumes of the centrifuged samples for assay were then added by the same means and the time of clotting, as indicated by the clumping of the Filter-Cel, recorded. Estimation of the end point was assisted by running the mixtures continuously up and down a fine Pasteur pipette.

RESULTS

Pathological Changes Following Insertion of Cellophane Sacs Intraperitoneally

Eighteen hours after sacs have been placed in the peritoneal cavity of laboratory animals, acute inflammatory changes are evident. The peritoneal cavity contains a small amount of serous exudate rich in polymorphonuclear leucocytes. The sac is embedded in a fibrinous cast gumming it to the omentum and intestines. Embedded in the fibrin are large numbers of polymorphs. Flakes of fibrin may be seen over the abdominal organs. The exudate is usually sterile, but may contain coliform bacilli or gram positive cocci particularly in mice. Provided precautions are taken in inoculating the sacs, it is rare to find infection due to organisms from the sac.

pH of Sac Contents

Dubos (1954) drew attention to the fall in pH in an area of inflammation. To test whether a fall in pH was sufficient to affect cultures inside the sac, sacs containing uninoculated saline were placed intraperitoneally in mice and rabbits for from 18 hr. to 3 days, and the pH of the saline measured electrometrically. In all cases the pH was between 7·6 and 7·8, showing no increase under these conditions.

Growth of Staphylococci in vivo

Table II shows the amount of growth *in vivo* in 24 hr. produced by various strains of staphylococci inoculated into saline in cellophane sacs. The number of organisms inoculated was about 10^5/ml. Except for 4 strains of coagulase negative staphylococci and the P80 strain of coagulase positive staphylococci in

TABLE II.—*Growth in 24 hr. of Strains of Staphylococci in Saline-filled Cellophane Sacs Placed in Peritoneal Cavities of Laboratory Animals*

Inoculum 10^5 organisms/ml.

Coagulase positive strains	Growth (mg.*/ml.) in				Coagulase negative strains	Growth (mg.*/ml.) in	
Strains	Mouse	Guinea-pig	Rabbit	Rat	Strains	Mouse	Rabbit
V8	1·0–1·3	1·3–2·4	0·83–1·76	1·1–7·0	S3	1·7	0·85
Newman	1·0–1·7	0·58	1·1–1·76	1·17	S92	1·5	0, tr
P80	1·35–1·76	1·8	0 or tr†	2·04	S294	1·3–1·55	tr (0·065)‡
Oxford	1·35–2·6	1·9	1·16	2·46	S295	1·3	0·87
12 strains from osteomyelitis	1·1–2·75		Not tested		346	1·0	0·84
5 non-haemolytic strains:					Air	1·0–1·15	tr (0·2)‡
A	0·58	Not tested	0·89	Not tested	29486	0·15	0
B	2·7	,, ,,	Not tested	,, ,,			
C	1·7	,, ,,	0·71	,, ,,			
D	3·7	,, ,,	0·28	,, ,,			
E	3·0	,, ,,	0·81	,, ,,			

* 1 mg. is approximately equivalent to 10^9 staphylococci.
† With inoculum 10^6/ml., growth of 0·65 mg./ml. was obtained; tr = trace.
‡ Figures in brackets denote growth in 48 hr.

rabbits, all strains grew well in all animals used. In general, growth in the rabbit was less than that in other animals. The mouse constituted a particularly good medium, and no significant difference was observed between the growth of coagulase positive and negative strains. Of the 4 coagulase negative strains which failed to grow in 24 hr. in the rabbit, 2 grew after 48 hr. When these were inoculated into a fresh sac and placed in a 2nd rabbit they grew overnight. The recovered strains seemed permanently adapted to the rabbit, for the ability for rapid growth was not removed by subculture on tryptic-heart agar. This observation suggests that a mutant adapted to grow in the rabbit was selected. Similar results were obtained with P80 (see below) which initially failed to grow in rabbits from an inoculum of 10^5 organisms but grew when the size of the inoculum was increased 10-fold (Table II). When retested in a fresh sac the strain grew overnight with an inoculum of 10^5 organisms/ml.

Growth Curves In Vivo

The rate of growth in sacs of three coagulase positive strains in rabbits, guinea-pigs and mice, and one coagulase negative strain in rabbits and mice, was now determined by viable counts. Empty sacs were placed intraperitoneally, and the next morning a known number of viable organisms in saline was inoculated into the sac. Samples were removed at intervals for viable counts until maximum growth was reached. Adjustments were made for loss of fluid by measuring the volume of the whole contents of the sac at the time the sample was taken. The results are shown in Figs. 3–7.

With the Oxford and V8 strains in all animals fairly regular results were obtained with a lag phase of 2 hr. and a log phase, representing some 10 generations, lasting about 6–8 hr., giving a mean generation time of about 30–40 min. The Oxford strain growing in the guinea-pig was somewhat slower with a mean generation time of 68 min. P80, although giving the same picture in the mouse as the other strains, (*i.e.* a lag period of 2 hr. and a mean generation time of 40 min.) grew very differently in the guinea-pig and rabbit. In the guinea-pig the lag phase was increased to 8 hr. with a slow transition to logarithmic growth, suggesting adaptation or selection of a mutant. In the rabbit, little or no growth was observed for some 24–30 hr. and growth thereafter was so irregular that no curve could be constructed, (Fig. 5). When the contents of the sac at 32 hr. (Fig. 5), after isolation and growth on TMA, were inoculated into a fresh sac in another rabbit, using the same size of inoculum, the passaged organisms grew after a lag of 4 hr. with a mean generation time of about 60 min. It appears that P80 in the rabbit, even more than in the guinea-pig, requires the selection of a more rapidly growing mutant. The coagulase-negative Air strain in mice grew well with a lag phase of 2 hr. and a mean generation time of 51 min., but in rabbits there appeared to be an exponential fall in count from $1{\cdot}57 \times 10^5$ cells inoculated to about 100 in 14 hr. (Fig. 7). However, after 24 hr., some growth occurred. Thus this strain resembles the P80 strain in requiring the selection of a mutant more adapted to the environment of the animal.

Comparison of growth curves in vivo *and* in vitro

It was of interest to compare these results with those found *in vitro*. In order to make valid comparisons, it was necessary to obtain conditions as near

as possible to those *in vivo*. Sacs containing uninoculated saline were placed in the peritoneal cavity of rabbits overnight, and their contents used as a culture medium for the growth of V8, Oxford and P80 in T tubes shaken in a water bath at 37°. No visible growth was found either in this medium alone or when supplemented with 2 per cent sodium lactate and 1 per cent glucose. However, such conditions are not a true comparison with those *in vivo*. *In vivo*, nutrients are

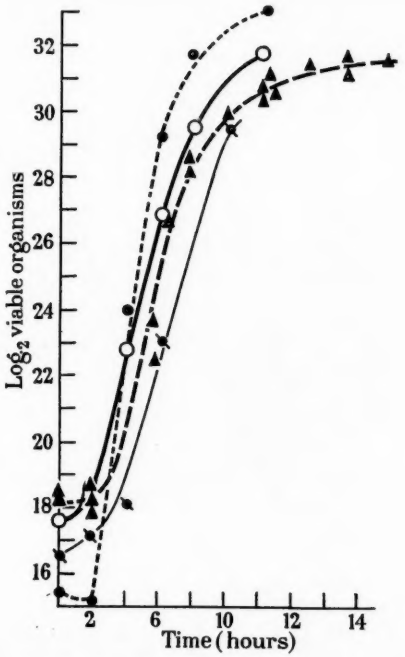


FIG. 3–7.—Growth curves of staphylococci in sacs *in vivo* and in serum diffusate *in vitro*.

FIG. 3.—Strain V8.

●- - - -● *in vitro* (serum diffusate).
○——○ in rabbit.
▲- - - -▲ in mouse (pool of 4 experiments).
●——● in guinea-pig.

continually being replaced through the walls of the sac as they are removed by the growing organisms, whereas in the *in vitro* experiments no such replacement takes place.

In order to make conditions more comparable, the organisms were grown in a saline-filled sac immersed in a relatively large volume of rabbit serum. For this purpose, the apparatus shown in Fig. 8 was used. A cellophane sac with nylon tube attached, as in the animal experiments, was placed through the open horizontal limb of a T tube, of $\frac{3}{4}$ in. diameter. Using a sewing needle, the nylon tube was passed through a rubber bung which fitted the open end of the limb,

and a No. 20 needle was inserted into its free end. The butt of the needle was closed with a plug as used in the animal experiments. The tube was autoclaved empty, and 10 ml. of Seitz-filtered fresh rabbit serum placed outside the sac. Two ml. of saline containing the same number as in the *in vivo* experiments (about 10^5/ml.) of viable staphylococci from an 18 hr. TMA culture was injected into the sac through the nylon tube. The culture was rocked in a water bath at 37° and samples removed at intervals for viable counts. As in the animal

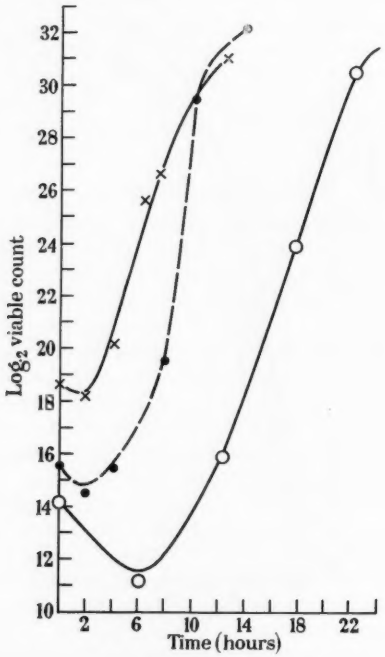


FIG. 4.—Strain P80.

●- - - -● *in vitro* (serum diffusate).
×——× in mouse.
○——○ in guinea-pig.

experiments, the volume was adjusted at the time of sampling for loss of fluid through the walls of the sac. The growth curves of V8, P80, Oxford and Air under these conditions have been superimposed on the curves showing the results *in vivo* (Fig. 3, 4, 6, 7). With all 4 organisms the total mass of growth was comparable to that obtained *in vivo*, and with V8 and Oxford there was also the same 2-hr. lag period. With P80, the lag phase extended over 4 hr. and there was the same suggestion of adaptation as shown by a slow transition to the log phase observed with the strain in guinea-pigs and rabbits. With the 3 coagulase positive strains, however, once growth was established, the mean generation time was significantly shortened (12–15 min.) compared with that found *in vivo* (30–60

min.). With the coagulase negative strain, Air, the results were similar, except that it grew more slowly (mean generation time 30 min.).

Since rabbit serum was used outside the sac, the results *in vitro* might be considered as directly comparable with the results in rabbits. However, there is a difference with all 4 strains, and this is particularly striking with P80 and Air

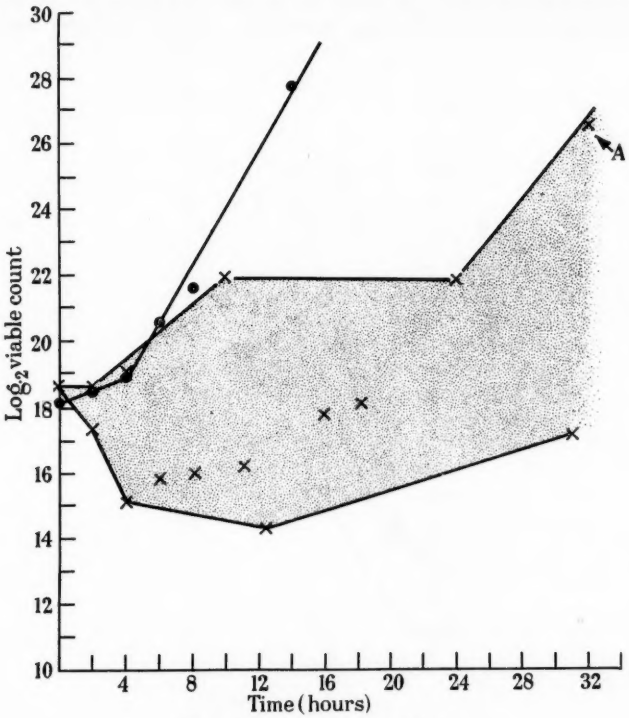


FIG. 5.—Strain P80 in rabbits

×——× not passaged.

●——● passaged once through sac.

Inoculum taken at point A and reinoculated into fresh sac. Hatched area includes points from 3 experiments.

strains. As mentioned above these strains in rabbits either refused to grow or required adaptation. Even after adaptation, P80 grew with a mean generation time prolonged to 60 min., whereas *in vitro* the mean generation time was 15 min.

Experiments to Determine the Cause of the Difference Between the Growth Curves In Vivo *and* In Vitro

In order to explain the difference between the growth curves *in vivo* and *in vitro*, particularly between those of P80 in the rabbit and in rabbit serum diffusate, the following factors were considered.

Temperature

The normal temperature of the rabbit is variable, the mean being about 39°. This is 2° higher than the temperature used for the experiments *in vitro*. In addition, the presence of the sac might have induced fever which would make the difference even greater. In order to test the latter point, the rectal temperature was taken at intervals over 24 hr. after the animal had recovered from the operation. The temperature did not rise above 39°, hence no fever was produced.

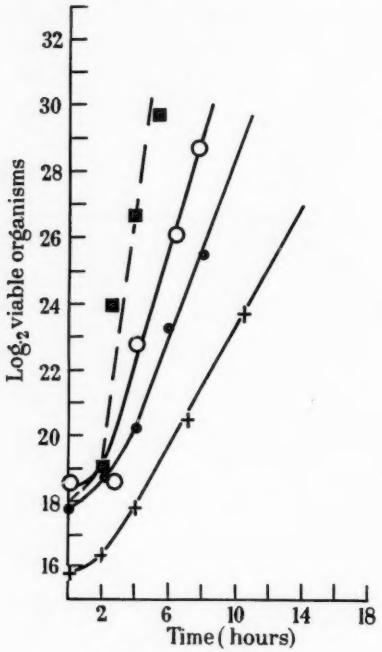


FIG. 6.—Oxford strain.

■- - - -■ *in vitro* (serum diffusate).
○——○ in mouse.
●——● in rabbit.
×——× in guinea-pig.

To make the *in vitro* cultures comparable with that *in vivo*, parallel cultures with P80 were run at 37° and 39° in serum diffusate *in vitro* using the cultural methods recorded in the last section. The results are shown in Fig. 9. Apart from the slightly increased lag phase at the higher temperature, only minor differences were found. The mean generation time at 37° was 17½ min. and at 39° 17 min.

Oxygen supply

It is likely that there was a considerable difference in the oxygen supply between cultures grown *in vitro* and those grown *in vivo*. *In vitro* the cultures

were shaken in air, whereas *in vivo* the atmosphere surrounding the sac was that of the abdominal cavity. Moreover, as has already been noted, there is an inflammatory reaction in the immediate vicinity of the sac. Such an area might well be deficient in oxygen (Dubos, 1954).

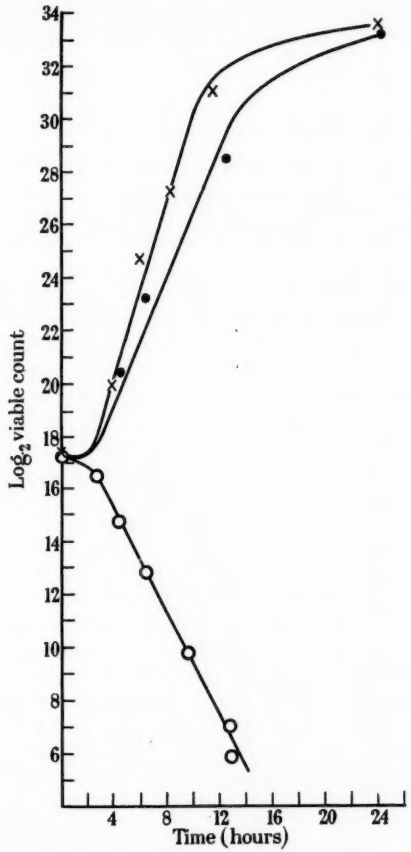


FIG. 7.—Air strain (coagulase negative).
×——× *in vitro* (serum diffusate).
●——● in mouse.
○——○ in rabbit.

Four cultures of P80 were put up in serum diffusate *in vitro* as in the previous experiment, but with different concentrations of air in the atmosphere surrounding the sac. The tubes contained 100 per cent air, 75 per cent air, 50 per cent air and 100 per cent nitrogen respectively. (The nitrogen, from a cylinder, was not burnt and therefore contained small quantities of oxygen). The various gas mixtures were made by placing the tubes in a desiccator, evacuating and filling

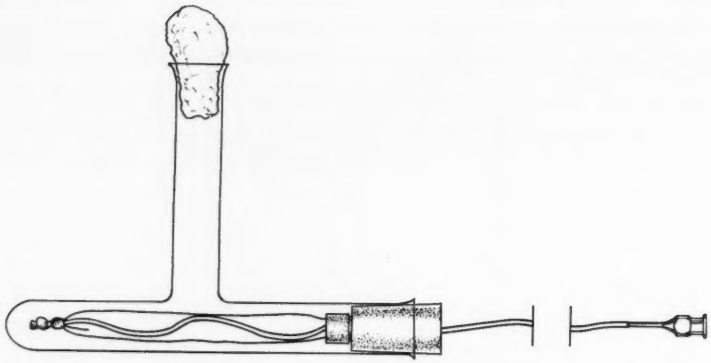


FIG. 8.—Apparatus for growth *in vitro* in serum diffusate. The sac contained 2 ml. saline and was immersed in 10 ml. rabbit serum. Inoculation and sampling were made through fine nylon tubes. The T-tube was continuously rocked during growth.

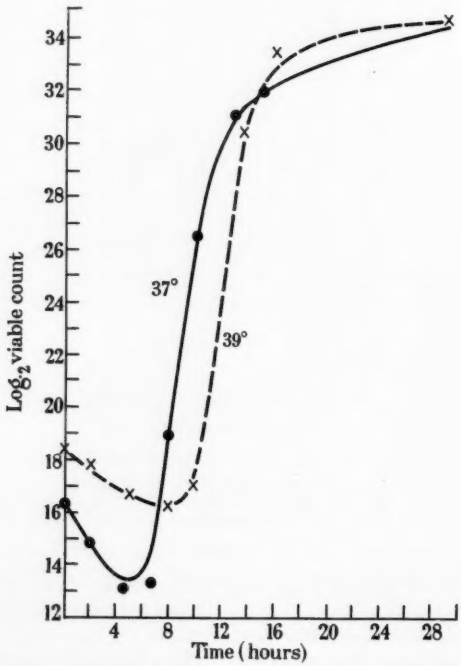


FIG. 9.—Growth curves of P80 *in vitro* in serum diffusate at 2 different temperatures.

with the appropriate gas mixture, removing the lid of the desiccator and immediately closing the tube with a rubber bung. Viable counts were made from the contents of the sac as in the previous experiments. There was no significant differences between the mean generation times of the 4 cultures. These were : in 100 per cent air 20 min., in 75 per cent air 22 min., in 50 per cent air 26 min. and in nitrogen 21·5 min. The difference in mean generation times between *in vitro* and *in vivo* cultures could not therefore be ascribed to differences in oxygen supply.

Differences in composition between inflammatory exudate and serum

The possibility that a difference in chemical composition between inflammatory exudate (*in vivo*) and serum (*in vitro*) might explain the differences in the growth curves, was tested by preparing an inflammatory exudate and using it in place of serum in *in vitro* cultures. The cells and fluid of the exudate were tested separately.

Tests with leucocyte extracts.—Extracts of leucocytes were prepared as follows : 200 ml. of saline was injected intraperitoneally into rabbits, and the following day the animals were killed and the peritoneal cavity washed out with saline. The cells, mainly polymorphs, were concentrated by centrifuging, counted in a haemacytometer and killed and largely broken up by repeated freezing and thawing. One ml. of the extract from 5×10^7 cells was added to 10 ml. of serum and a culture of P80 strain set up as in previous tests and compared with a similar culture without cells. Viable counts were made at intervals and growth curves plotted. Both cultures grew well with a mean generation time of 22–24 min. The inflammatory cells were thus not responsible for the difference observed *in vitro* and *in vivo*.

That the leucocyte extract contained factors inhibiting the growth of staphylococci was clearly shown when the extract was added to the saline within the sac and cultured with serum outside the sac. There was a rapid fall in count with both V8 and P80 strains from $1{\cdot}5–1{\cdot}8 \times 10^5$ to less than 100 cells in 8 hr. These inhibitory factors are apparently too large to pass through the wall of the sac.

Tests with the fluid from inflammatory exudate.—Pleural exudate was prepared by injecting rabbits intrapleurally with 10 ml. 0·2 per cent DNA (prepared from calf's thymus by Dr. Woodin). After 18 hr. the animals were killed, and about 4 ml. of turbid pleural fluid containing many polymorphonuclear leucocytes and red cells was removed from each animal, pooled and centrifuged free from cells. The material was a clear-straw-coloured fluid. Ten ml. of this fluid without filtration was used in place of serum in a T tube culture as in previous experiments. The saline inside the sac was inoculated with P80 (10^5/ml.).

The early part of the growth curve is shown in Fig. 10. The curve is quite different from that with serum diffusate. The lag phase is prolonged to about 6 hr., and there is a slow transition to the log phase and a mean generation time of about 1 hr., compared with a lag of 4 hr. and a mean generation time of about 26 min. in serum diffusate. The inhibitory effect of the inflammatory fluid was on the rate, rather than on the total mass, of growth, since in 31 hr. no difference in the amount of growth was observed whether the sac was immersed in pleural fluid or in serum. Since the exudate had been prepared by injecting DNA, the above experiment was controlled by testing for an inhibitory effect of 0·2 per cent

DNA on the growth of P80. No inhibitory effect was observed either on rate or mass of growth.

Test for lysozyme activity in pleural exudates and in sac contents

Since lysozyme has a molecular weight of about 15,000 and is known to pass through the walls of a cellophane sac (Roberts and Wells, 1938) it is possible that the observed inhibitory effect of pleural fluid is due to the presence of lysozyme. Although lysozyme is not known to inhibit the overall growth of coagulase positive

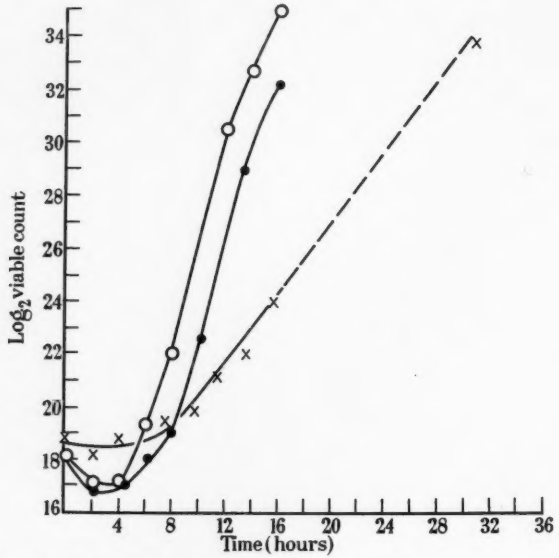


FIG. 10.—Growth curves of P80 *in vitro*:
×———× in a diffusate of rabbit pleural exudate (broken line no intermediate observation made).
○———○ in a diffusate of serum containing M/30,000 lysozyme.
●———● in a diffusate of serum with no addition.

staphylococci, its presence might modify the growth curve and explain the delayed growth of P80 in a diffusate of pleural exudate. That pleural fluid has lysozyme activity is shown by the inhibition of growth of *Micrococcus lysodeikticus* in a plate culture (Fig. 11, A). Pleural fluid diffusate also has this activity, but to a less extent (Fig. 11, B) showing that the factor responsible for the inhibition is diffusible. However there are, of course, factors in animal tissues inhibitory to *M. lysodeikticus* other than lysozyme.

Tests with lysozyme.—To test whether the inhibitory effect of the pleural fluid is due to lysozyme, lysozyme in a final concentration of 1/30,000 (a concentration shown to give a zone of inhibition on a plate culture of *M. lysodeikticus* of the same size as that produced by pleural fluid (Fig. 11) was added to 10 ml. of rabbit serum in a T tube, and a sac culture made as in the previous tests. A

control without lysozyme was included. Viable counts on both cultures were carried out and growth curves plotted. These are shown in Fig. 10. Both cultures grew well with a mean generation time of about 24 min. no significant differences being observed between them.

The culture supernatant of a sample from the serum-lysozyme diffusate after 6 hr. was tested for lysozyme activity by placing it in a well in the centre of a bulk-seeded agar plate culture of *M. lysodeikticus*. No inhibition of growth was observed showing that, under the conditions of the experiment, lysozyme had not passed through the sac. A similar test on the serum outside the sac at the end of the experiment showed that no loss of lysozyme had taken place. From this result it is clear that the inhibitory effect of pleural fluid diffusate on *M. lysodeikticus* (Fig. 11) and its inhibitory effect on the growth of P80, observed in the earlier experiments, are not due to lysozyme which, under the conditions of the experiment, failed to pass through the wall of the sac.

Further experiments to determine the nature of the factor responsible for the inhibitory effect are being carried out.

The Production of α Haemolysin and P.V. Leucocidin In Vivo

Estimations of α haemolysin and P.V. leucocidin were made from cultures from sacs placed intraperitoneally in laboratory animals. Parallel estimations were made from cultures grown in serum diffusate *in vitro* under the conditions described in the first part of this paper.

α Haemolysin

Saline in sacs placed in the abdominal cavities of mice, rabbits, guinea-pigs and rats, was inoculated with 10^5/ml. of V8, P80, Oxford and Newman and left in the animal for 24 hr., (except P80 in the rabbit, which owing to slow and irregular growth was left for 41 hr.). Similar sacs were suspended in rabbit serum, using the apparatus shown in Fig. 8, and grown *in vitro* (serum diffusate culture). The growth of both *in vivo* and *in vitro* cultures was estimated by Spekker absorptiometer and the culture assayed for α haemolysin. The results were expressed in Lh/mg of bacterial growth (Fig. 12). Although all cultures grew well, no α haemolysin was produced in serum diffusate *in vitro*. On the other hand *in vivo*, high yields were obtained in all animals, particularly in the rat, and with all strains. The production of α haemolysin during growth using V8 in the rabbit was followed by taking samples at 2-hr. intervals after inoculation and assaying for the amount of growth and haemolysin production. The course of production per unit growth appeared to lag slightly behind that of growth, reaching a maximum some 6–8 hr. after maximum growth and then declining sharply. This is in contrast to the course of production under the best conditions in *in vitro* (Elek, 1959) in which production is maximum after growth has ceased.

Test for mutants in sacs in vivo.—The possibility that the *in vivo* conditions selected a mutant particularly adapted to the production of α haemolysin was tested by plating cultures of V8, P80 Oxford and Newman from sacs in mice and rabbits onto nutrient agar containing 2 per cent washed rabbit red cells, incubating in 20 per cent CO_2 and noting the size of the zone of haemolysis. High titre antitoxic serum was spread over half the plate as a control. No differences were noted in the size of zones compared with similar platings of the parent strains.

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Non-haemolytic coagulase positive strains.—Five coagulase positive strains freshly isolated from infective lesions produced no α haemolysin on a plate containing 2 per cent washed rabbit cells, although δ haemolysin was produced by 4 of them. Also, no soluble α haemolysin was found when the strains were cultured on 0·2 per cent nutrient agar in 20 per cent CO_2. When they were inoculated into saline-filled sacs in mice and rabbits, three of them produced 0·25, 0·44 and 0·27 Lh/mg. of growth in mice, and one of these produced as much as 5·4 Lh/mg. in rabbits. The results could not be explained by the selection of variants, since no α haemolysin was produced when these strains were recovered and tested *in vitro*.

Coagulase negative strains.—The seven strains tested were non-haemolytic as shown by the absence of zones of haemolysis on rabbit blood agar plates incubated

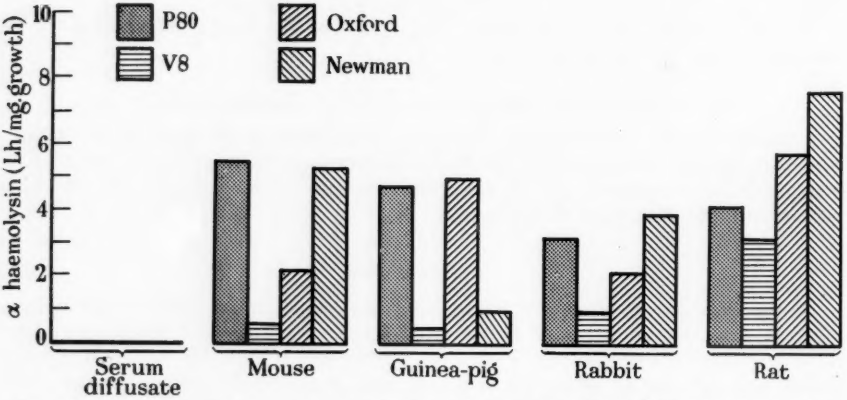

FIG. 12.—The production of α haemolysin by P80, V8, Oxford and Newman strains of staphylococci in sacs in serum diffusate *in vitro* and in the peritoneal cavities of various laboratory animals.

in 20 per cent CO_2 in air. Of the 7 strains, 6 grew well in sacs in mice (Table I) but none produced any α haemolysin ($< 0{\cdot}01$ Lh/mg.). Tests on the cultures of the 4 strains which grew in sacs in rabbits (Table I) were also negative.

Tests to determine the cause of the difference in haemolysin production under in vivo *and* in vitro *conditions*

As with the experiments to determine the difference in growth curves in the first part of this paper, the following factors were considered.

Temperature.—P80 was grown in rabbit serum diffusate for 24 hr. as in the previous test, but the temperature of the water bath was raised to 39°. No α haemolysin was produced, so that the difference could not be attributed to this factor.

Oxygen supply.—Cultures of V8 grown for 24 hr. in different concentrations of air in nitrogen in serum diffusate described on p. 323 were centrifuged and the supernatants tested for α haemolysin. No haemolysin was found.

Pleural fluid diffusate.—The culture of P80 in rabbit pleural fluid diffusate after 31 hr. (p. 326) was centrifuged and tested for α haemolysin. No haemolysin was found. Thus, although factors in pleural fluid may explain the difference between the growth of P80 *in vitro* in serum diffusate and that *in vivo*, they do not explain the difference in haemolysin production.

The Production of P.V. Leucocidin

The early experiments were carried out before Woodin (1959) showed that the leucocidal activity of culture filtrates was due to 2 synergistic proteins "Fast" and "Slow". Strain V8, P80 and Oxford were grown in saline-filled sacs in the abdominal cavities of mice, rabbits, guinea-pigs and rats and the cultures assayed for leucocidin. High yields were obtained, in many cases several fold greater than the amounts produced under the best conditions *in vitro*.

When Dr. Woodin provided us with purified samples of Fast and Slow components, each was assayed separately in cultures from sacs *in vivo* in the presence of excess of the other as described under "Methods".

Assays were made on 24-hr. cultures of V8, Oxford and Heath in mice, rabbits and guinea-pigs and of P80 in mice and guinea-pigs, and on 41-hr. cultures of P80 in rabbits (since the strain grows slowly in rabbits). Cultures were also put up *in vitro* in T tubes containing yeast-casein hydrolysate medium (Gladstone and van Heyningen, 1957) as modified by Woodin (1959) and shaken overnight at 37°. These are the conditions found to be optimal for the production of leucocidin *in vitro*. Cultures were also put up in sacs in rabbit serum diffusate *in vitro*. The assays were in all cases related to L+/mg. growth.

Fig. 13 shows that in all cultures *in vitro* and *in vivo* both fast and slow components are produced, the latter in amounts approximately 5–10 times greater than the former. This is a constant finding and is due to a difference in size between the 2 arbitrary fixed units of anti-F and anti-S in the Standard serum CPP 76/63 (Woodin, unpublished). It is clear that both factors are produced in amounts far greater *in vivo* than under the best conditions *in vitro*. Negligible amounts are produced in serum diffusate *in vitro*. As with α haemolysin, different conditions for growth were tried in these cultures: the temperature was increased to 39°, cultures were grown under lowered oxygen tension, and pleural fluid was used in place of serum. In no case was the amount of either F or S increased.

Coagulase positive strains producing none or negligible amounts of P.V. leucocidin in vitro.—Using the best conditions yet known for the production of P.V. leucocidin *in vitro* as described above, 7 strains of coagulase positive staphylococci (freshly isolated by Dr. A. G. Towers from osteomyelitis) were tested for the production of leucocidin. Five produced less than 0·01 L+/mg. leucocidin and 2 less than 0·1 L+/mg. When these strains were placed in sacs in the peritoneal cavity of mice, and the culture fluid after 18–24 hr. growth tested for leucocidin, 6 were found to have produced 0·53–2·9 L+/mg. and one 0·05 L+/mg. As with the increased production of α haemolysin *in vivo*, there was no evidence that a mutant had been selected. It would appear that the environment *in vivo* is suited to the production of both α haemolysin and P.V. leucocidin. One coagulase positive strain, Newman, appears to produce no P.V. leucocidin under any condition. Both *in vivo* and under optimal conditions *in vitro* less than 0·01 L+/mg. was obtained.

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Coagulase negative strains.—The 7 strains tested failed to produce any leucocidin (< 0·01 L+/mg.) in sacs *in vivo*.

Coagulase

Estimations of free coagulase were made on the supernatants from 18–hr. cultures of V8, P80, Oxford and Newman in saline filled sacs in mice and rabbits. Except with Newman, which clotted plasma within 1 min., no coagulase was found. However, it is known that the maximum production of coagulase takes place in the lag phase and thereafter coagulase may be rapidly destroyed (Duthie,

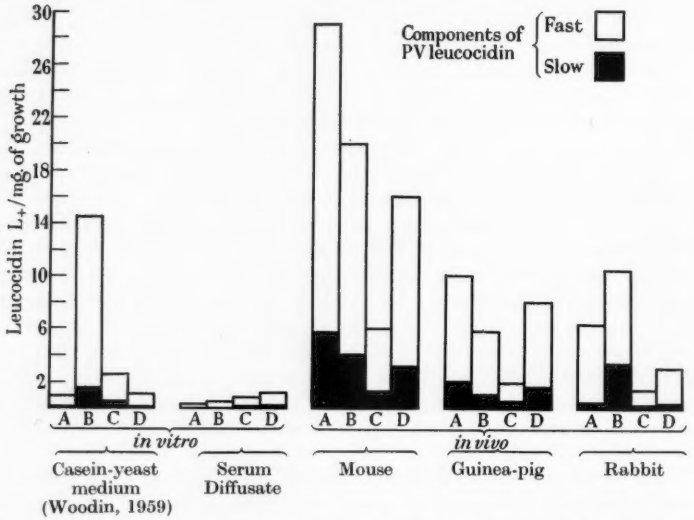

FIG. 13.—The production of S and F components of P.V. leucocidin by P80, V8, Oxford and Heath strains of staphylococci *in vitro* and *in vivo* in various laboratory animals.
A, P80; B, V8; C, Oxford; D, Heath.

1954). Tests on the supernatants in the lag phase were accordingly carried out as follows:—Four uninoculated saline-filled sacs, and similar sacs inoculated with V8, P80, Newman and Oxford, were placed in the peritoneal cavities of mice and rabbits. After 18 hr. the fluid from all sacs was withdrawn. The uninoculated sac contents were diluted with the four grown cultures to give a 10 per cent inoculum and reinjected into the empty sacs from which they had been removed. Samples were removed after 2 and 5 hr. and the supernatants estimated for free coagulase.

The Newman strain produced appreciable amounts of coagulase in both species of animal (clotting time less than 1 min.), V8 produced none in either species and P80 and Oxford only in mice (clotting time 7 min. and 1 hr. respectively). There was evidence that coagulase produced by P80 was rapidly destroyed.

DISCUSSION

The experiments recorded in this paper show that strains of coagulase positive staphylococci grow well *in vivo* when protected from phagocytes and antibacterial factors greater than *c* MW 20,000 by being enclosed in cellophane sacs.

By a simple sampling device, it has been possible to trace the growth curves from the time of inoculation and to compare them with those of cultures grown in serum diffusate *in vitro*. Using 3 coagulase positive strains P80, V8 and Oxford, certain differences were noticed. The lag phase is usually prolonged, but the most constant feature is the increase in the mean generation time during the log phase which may be more than double that *in vitro*. The differences are more marked in the rabbit and guinea-pig than in mice, and with P80 than with V8 and Oxford. P80 in the rabbit on primary inoculation may not grow unless at least 10^6 cells/ml. are inoculated, and even after passage, grows with a generation time three times that in a diffusate of rabbit serum *in vitro*. These differences are probably related to the inflammatory reaction which is produced in the vicinity of the sacs in the peritoneal cavity, since the same prolonged mean generation time is obtained in sacs *in vitro* when rabbit serum outside the sac is replaced by rabbit inflammatory pleural exudate. There is evidence that pleural exudate contains diffusible factors, not found in serum, which inhibit the growth of *M. lysodeikticus*, and it is possible that such factors are also responsible for the modified growth curve of P80 in a diffusate of pleural fluid and *in vivo*. Lysozyme, however, although it is said to be present in large amounts where inflammatory cells accumulate (Moeller, Marshall and Kirsner, 1951), is not one of these factors, since it failed to pass into the sac when added to serum outside the sac in *in vitro* cultures.

Dubos (1954) has emphasised the inhibitory action on staphylococci of lactic acid produced in an area of inflammation. However, this also cannot be the factor concerned here, since repeated estimation of the pH inside the sac both *in vivo* and *in vitro* cultures, always gave values of 7·6–7·8. The factor concerned is being further investigated.

Coagulase negative strains grow readily in cellophane sacs in mice, and in rabbit serum diffusate *in vitro*. However, *in vivo* in rabbits, 4 out of 7 failed to grow, or grew very feebly. Viable counts at intervals on one strain, Air, showed a rapid destruction of the organisms, but a few survived and a more resistant mutant appeared to be selected. As with P80, the inhibitory effect of the *in vivo* environment on these strains is likely to be related to inhibitory factors in inflammatory fluid. Certainly inflammatory pleural exudates contain diffusible factors which inhibit the growth of these strains. Ekstedt (1956) has described a factor in human serum inhibitory to the growth of coagulase negative, but not coagulase positive, strains of staphylococci. This factor, however, is closely associated with the serum proteins and is not diffusible and cannot therefore be the factor concerned here.

Although *in vitro* the optimal conditions for the production of α haemolysin and the " slow " and " fast " components of P.V. leucocidin are very different, the former requiring prolonged growth usually in an atmosphere of 20 per cent CO_2 (see Elek, 1959) and the latter being produced early in growth (Woodin, 1959), *in vivo* both toxins are produced under the same conditions. Moreover, the amounts produced per unit weight of organism often far exceeds the amount

produced under the best conditions *in vitro*. Of particular interest is the observation that strains which fail to produce α haemolysin or P.V. leucocidin *in vitro*, may do so in sacs *in vivo*. Thus 3 out of 5 strains which failed to produce α haemolysin *in vitro* produced appreciable amounts *in vivo*. Similarly, of 6 strains producing negligible amounts of P.V. leucocidin under the best conditions *in vitro*, 5 produced appreciable amounts *in vivo*. It is not uncommon to find coagulase positive strains described which are said to be deficient in α haemolysin production (Lack and Wailling, 1954) and P.V. leucocidin (Gladstone and van Heyningen, 1957). It is possible that many of these strains would be found to produce these toxins if tested *in vivo*. However, some coagulase positive strains appear to be absolutely deficient in the ability to produce one or other of these toxins under any conditions of culture. In the present work, 2 strains failed to produce any α haemolysin and 1 strain, Newman, failed to produce either factor of leucocidin. No strain has yet been found to produce only one factor of leucocidin.

Even when α haemolysin and both factors of leucocidin are produced in large amounts *in vivo*, neither toxin is produced in serum diffusate *in vitro*. Some diffusible factor or factors stimulating the production of these toxins must therefore be present in the animal but not in its serum. Such factors are also not present in inflammatory pleural fluid, since neither α haemolysin or P.V. leucocidin was obtained in a diffusate of pleural fluid *in vitro*. The nature of these factors has still to be determined.

In contrast to α haemolysin and P.V. leucocidin, the production of coagulase does not appear to be enhanced by growth *in vivo* under the experimental conditions described in this paper.

SUMMARY

A total of 21 coagulase positive and 7 coagulase negative strains of staphylococci have been grown in cellophane sacs intraperitoneally in laboratory animals. One coagulase positive strain, a phage type 80, and 4 coagulase negative strains gave little or no growth in rabbits, but all strains grew well in mice. All of 4 coagulase positive strains tested in guinea-pigs and rats also grew well. The growth curves *in vivo* of 3 coagulase positive strains were compared with similar curves *in vitro* in rabbit serum diffusate. The mean generation times *in vivo*, particularly with P80 strain, were 2-fold or greater than those *in vitro*. A more rapidly growing mutant can be selected. Evidence is presented that these differences are due to a diffusible inhibitory factor or factors present in inflammatory exudate and absent from serum. This factor is not lysozyme.

High yields of both α haemolysin and the " Fast " and " Slow ' components of PV leucocidin are produced *in vivo*, but not in serum diffusate or diffusate of inflammatory exudate *in vitro*. Some strains, which fail to produce these toxins under optimal conditions *in vitro*, do so *in vivo*. This is not due to the selection of toxigenic mutants. It is pointed out that tests for toxigenicity on cultures grown *in vitro* may not always be valid, and it is suggested that in the past strains have been falsely labelled as non-toxigenic. Some strains produce coagulase *in vivo*, but the yield is no greater than *in vitro*.

We are indebted to Dr. A. M. Woodin for samples of pure " F " and " S " components of leucocidin, to Dr. A. G. Towers and Dr. R. J. Dubos for cultures

of staphylococci, to Dr. Mollie Barr for staphylococcal antitoxin and to Miss J. Tyrrell and Mr. R. J. Smith for technical assistance.

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SURVEY OF PAPERS

STOREY gave cortisone to rats fed on diets which were " normal ", deficient or unbalanced as to calcium, phosphorus and vitamin D. He studied bone changes in the rats histologically. Cortisone caused the development of dense metaphyseal bone on " normal " diets, and this was accentuated by vitamin D deficiency. With reduction in the amount of, or alteration in the ratio between calcium and phosphorus in the diet, cortisone caused osteoporosis (p. 207).

NAIRN, CHADWICK and FRASER have purified porcine renin by a combination of methods including electrophoresis, adsorption and immunological precipitation. The products have been examined by gel diffusion analyses (p. 214).

SOMMERVILLE measured the rate of adsorption to monkey kidney cells of 4 ECHO virus types—1, 2, 7 and 11. He used 3 methods—titration of unadsorbed inoculum, counting of plaques, and observing time to degeneration of cells after varying periods of contact between virus and cells. All these methods showed that the viruses adsorbed in this order of efficiency :—7, 1, 11, 2, and he thinks that the differing rates of attachment of these viruses to host cells explain differences in their plaque producing potential (p. 222).

SOMMERVILLE wished to determine why ECHO type 2 virus, which forms plaques in monkey kidney cell monolayers in liquid medium, failed to do so under agar neutral red overlays. Testing separately overlay constituents not used in the liquid medium, he found neutral red inhibited plaque formation (p. 229).

NORTH and HAZEL DOERY have examined certain snake venoms and bee venom for antagonism to the action of staphylococcal α-toxin in mice. They show that the venom of the tiger snake protects against the dermonecrotic effect of the toxin when given subcutaneously. This venom and others containing phosphatidase A protect mice against the lethal effect of intravenous challenge with the toxin. They are less effective against subcutaneous challenge and quite ineffective against intraperitoneal challenge. Further they show that the effectiveness of the antagonism of these venoms to the toxin is increased by toxoiding with formalin (p. 234).

HAZEL DOERY and NORTH, continuing their work on the antagonism of certain snake venoms to staphylococcal α-toxin, have fractionated the venom of the black snake and examined the antagonistic effect of the fractions. They found that the antagonistic effect is proportional to the amount of phosphatidase A. They further examined the products of the action of this enzyme upon phosphatides. They found that the lysolecithins tested protected mice against the effects of the toxin, but only unsaturated fatty acids inactivated its effect. They suggest that phosphatidase A and other enzymes play a part in the natural defence mechanism against bacterial toxins (p. 243).

HOBSON has investigated the effect of administering cortisone to mice previously infected with *Salm. typhi murium*. He found that it enhanced the infectivity by allowing the critical population of organisms to be attained more rapidly ; that there was a lag period before cortisone exerted its full effect on the host parasite relationship and that its effects in this respect were much less pronounced in animals that were chronic carriers (p. 251).

BOYD and SMITH examined the beds of autografts and homografts in rats at the onset of graft necrosis, both histologically and by determination of histamine levels. Donors and recipients were used untreated, or after either or both had received histamine, antihistaminics

or the histamine liberator substance 48/80. Depletion of skin histamine, however achieved, delayed the healing of autografts and prolonged the life of homografts. There was some difference between the injected agents in their effect upon mast cells in the dermis (p. 259).

BARBARA HESLOP, IRMGARD ZEISS and NISBET implanted in Wistar rats bone autografts or homografts from hooded donors. The implants were lengths of tibia denuded of periosteum and medullary tissues ; they were put between the dermis and the panniculus carnosus muscle. They found, by histological examination of grafted areas at intervals up to 250 days, that the maximum cellular response came later and lasted longer round homografts than round autografts, and that osteogenesis and connective tissue formation were much more vigorous round autografts. They also noted the extent to which implanted osteocytes survived (p. 269).

BROGAN has examined the biochemical properties of a water-soluble complex obtained from sputum by ultrasonic vibration. Components of the complex have been identified and suggestions have been made for the origins of these components (p. 288).

ÉVA HORVÁTH, SÓLYOM and KORPÁSSY poisoned rats with tannic acid, using one subcutaneous dose of 250 mg./kg. body weight, which was not lethal. They examined the rats' livers histologically and chemically and analysed their serum proteins at intervals up to 7 days after poisoning. Their results led them to conclude that the action of tannic acid on the liver is different from that of most hepatotoxic substances, but its mechanism cannot yet be defined (p. 298).

SMITH and MILES caused peritoneal inflammation in rats by injecting bacteria or bacterial toxin. Protein exuded into the peritoneal fluid was measured spectrophotometrically and expressed as optical density per unit volume. Protein begins to be exuded in quantity 3–4hr after the injection of the noxious agent. Measurements of histamine exudation, and records of the effect of added histamine, histamine liberator (48/80) and the antihistaminic triprolidine on protein exudation, together with microscopy of mesenteric tissue mast cells, convinced the authors that histamine was not essential to the early reactions of acute infection (p. 305).

GLADSTONE and GLENCROSS have grown staphylococci in cellophane sacs in the peritoneal cavity of rabbits, guinea-pigs, mice and rats. They plotted the growth curves of the strains and estimated their production of α-haemolysin, P.V. leucocidin and coagulase. They found that the mean generation times *in vivo* were 2-fold or greater than those *in vitro* ; this being particularly true of a phage type 80 strain. Evidence is produced that this was due to a diffusible inhibitory factor present in inflammatory exudate, but absent in serum.

High yields of α-haemolysin and P.V. leucocidin were produced *in vivo* and some strains produced these toxins *in vivo* but not *in vitro*, suggesting that tests for toxigenicity performed on cultures grown *in vitro* may not always be reliable (p. 313).

ERRATUM

Vol. XLI, No. 3, June 1960.

(G. P. Gladstone and E. J. G. Glencross)

P. 330. Fig. 13. Key should read: Slow □
Fast ■